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**GAMETE AND CELL TECHNOLOGIES FOR USE IN  
BIOLOGICAL RESOURCE BANKING**

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
In partial fulfillment of the  
Requirements for the degree of  
Doctor of Philosophy

in

The Interdepartmental Program of  
Animal Sciences

by  
Liesl Nel-Themaat  
B.Sc., Stellenbosch University, 2000  
May 2006

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## LIST OF ABBREVIATIONS

AI.....	Artificial insemination
ART.....	Assisted reproductive technologies
BRBs.....	Biological resource banks
CCB.....	Cytochalasin B
CSE.....	Cryoprotectant semen extender
COC.....	Cumulus-oocyte complex
DMAP.....	6-dimethylaminopurine
DMEM.....	Dulbecco's modified Eagle medium
DMSO.....	Dimethyl sulfoxide
ET.....	Embryo transfer
FBS.....	Fetal bovine serum
FITC.....	Fluorescein isothiocyanate
FSH.....	Follicle stimulating hormone
GCN.....	Gulf Coast Native
GRB.....	Genome resource banking
ICM.....	Inner cellular mass
ICSI.....	Intracytoplasmic sperm injection
igNT.....	Intergeneric nuclear transfer
IVF.....	<i>In vitro</i> fertilization
IVM.....	<i>In vitro</i> maturation
LN <sub>2</sub> .....	Liquid nitrogen
NCS.....	New born calf serum
NT.....	Nuclear transfer
PAOs.....	Parthenogenetically activated oocytes

PBS.....	Phosphate-buffered saline
PESA.....	Percutaneous epididymal sperm aspiration
PF.....	Paraformaldehyde
PVP.....	Polyvinyl pyrrolidone
RT.....	Room temperature
SC.....	Somatic cells
SCNT.....	Somatic cell nuclear transfer
SE.....	Standard error
TB.....	Trypan blue
TGF- $\beta$ .....	Transforming Growth Factor-Beta
TPD.....	Trophectoderm
TRT.....	Treatment
TUGA.....	Transvaginal ultrasound-guided oocyte aspiration
$\alpha$ -MEM.....	Minimum essential medium alpha medium

## ABSTRACT

Biological resource banking is becoming important for endangered species conservation. A series of experiments were conducted to address issues concerning collection and utilization of biomaterials from Gulf Coast Native (GCN) sheep (model species) (*Ovis aries*) and eland antelope (*Taurotragus oryx*). In the first experiment, two ejaculates were collected 10 minutes apart from each of five rams three times a week for three weeks to maximize output and minimize handling time. Semen volume, concentration and total number of spermatozoa were significantly greater in first ejaculates, whereas, pre-cooled, cooled and post-thaw motility, as well as sperm survival, were greater in second ejaculates. The second experiment was designed to develop a practical method for freezing skin biopsies for tissue culture. Cell survival was enhanced by an equilibration time of at least 15 minutes in biological medium (containing blood or serum) as compared to nonbiological medium (containing PVP). Then, the possibility of using semen and cooled milk as sources of somatic cells (SC) for *in vitro* culture was evaluated. Fresh, cooled and cryopreserved semen from rams and fresh eland semen was cultured and although SC plated, proliferation was low. From milk, cell attachment was observed in 92% of the samples, whereas, only 38% proliferated in culture. Therefore, the ensuing experiment was focused on increasing *in vitro* proliferation of semen-derived SC by developing (1) a Percoll gradient technique to separate SC from spermatozoa before culture and (2) a method for co-culture of isolated SC with inactivated mouse fibroblasts. Proliferation was significantly increased by co-culture, but contact between the semen-derived SC and feeder cells was not necessary. Finally, intergeneric nuclear transfer (igNT) of semen-derived eland epithelial cells into bovine cytoplasts showed that these cells could direct early embryonic development up to the 8-cell stage. Determination of BrdU-incorporation and evaluation of nuclear status

revealed that initial remodeling of the eland nucleus was similar to that of bovine NT embryos within the first 16 hours post-activation. However, after 84 hours, only 13% of interspecies embryos had cycling nuclei in their blastomeres. Future improvements in the technology should eventually allow cloned offspring to be produced from semen-derived somatic cells.

# CHAPTER 1

## INTRODUCTION

The International Union for Conservation of Nature and Natural Resources (IUCN) red list of 2004 contains more than 5,000 vertebrates and almost 2,000 invertebrates.

Approximately 20% of mammalian species are threatened with extinction and the numbers have been increasing consistently over the past decade.

For some critically endangered species, the captive population accounts for most of the surviving individuals. Thus proper genetic management becomes even more important and breeding recommendations may include pairing of animals that are sexually incompatible or geographically distant. In such cases, assisted reproductive techniques (ART) such as artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and embryo transfer (ET) may be the preferred method of procreation.

Numerous publications have discussed the role of assisted reproductive science in conservation (Wildt and Wemmer, 1999; Comizzoli et al., 2000; Pukazhenth and Wildt, 2004) and they explicitly stated that the ultimate utility of ART as an approach for propagation of endangered species depends on the maximal availability of potential sources of genetic diversity. Therefore, the ability to cryopreserve semen, oocytes, embryos, cell lines and tissues assumes an essential role in multifaceted conservation programs that include ART among its components.

The successful cloning of an adult animal (Wilmut et al., 1997) broadened the ART horizon far beyond what had previously been considered to be within the realm of possibility. Many genetically valuable individuals are incapable of, or have never had the opportunity to reproduce, resulting in permanent loss of their genes. However, adult somatic cell nuclear transfer (NT) provides an opportunity to introduce new genes into genetically depleted



populations. Such potential has provided substantial motivation for expansion of efforts to conserve bio-materials by cryostorage.

Biological resource banks (BRBs) typically contain germplasm, embryos and cell lines specifically for use with ART. In addition, they often are repositories of blood, tissue, urine and fecal samples that are useful for determining hormone patterns, nutritional status, medical condition and genetic fitness (Wildt and Rall, 1997; Wildt, 2000).

A recent example in which the combination of BRBs and ART could have had a positive influence on the gene pool is that of the Black-footed ferret (*Mustela nigripes*). The species is a native of the western United States that nearly became extinct in the 1980s due to a combination of human interference with its habitat and an epizootic outbreak of canine distemper (Seal et al., 1998). If gametes and embryos had been collected and cryopreserved before the population size plummeted, much of the genetic diversity would have been retained for subsequent re-introduction into new generations (Wildt et al., 1997). By learning from such experiences, we could potentially prevent future genetic losses in other species by collecting and storing biomaterials while the diversity is still available.

Therefore, BRBs are valuable as an augmentary source of genetic material that, through the use of ART, can potentially enhance variation in small, isolated gene pools depleted of diversity. The potential role of BRBs is only limited by our capability to re-incorporate the bio-material into the extant population. Therefore, expanding and improving the efficiency of novel biotechnologies are critical determinants of their ultimate utility for assisting in preventing extinction of endangered species.

The current study was conducted to develop methods to collect, bank and utilize biological materials for conservation purposes. In Chapter 3, the effect of cryopreservation medium, equilibration time and tissue type on the success of deriving cell lines from flash-frozen sheep tissue biopsies was evaluated to develop a practical method for field biologists to obtain viable genetic samples. Then, in a subsequent chapter, the effect of multiple electroejaculation

procedures during a single session on semen output and quality in Gulf Coast Native (GCN) sheep was evaluated. Somatic cells isolated from milk and ejaculated semen are potential sources of donor cells and should be included in genomic resource banks for endangered species. Accordingly, Chapters 5 and 6 consist of experiments on isolation, culture and characterization of somatic cells from sheep milk and from semen of GCN sheep and Eland antelope. In addition, optimization of the culture system for isolated epithelial cells is described. Finally, in Chapter 7, the ability of eland semen-derived epithelial cells to dedifferentiate in bovine cytoplasts and support early *in vitro* embryonic development after intergeneric NT is presented.

## **CHAPTER 2**

### **LITERATURE REVIEW**

#### **2.1 Cryopreservation for Biological Resource Banking**

Cryopreservation of gametes, embryos, tissues and cell lines could play an important role in ART for conservation purposes. It would be extremely challenging to successfully perform AI, IVF, ET, ICSI or NT if all biomaterials used were fresh. The limited number of donors and/or female recipients, the short life span of fresh semen, small window of opportunity for AI or ET in recipient females (Evens and Maxwell, 1987; Gordon, 1994a, 1994b) and the fact that cells in culture reach senescence after prolonged culture (Kill and Faragher, 2000), all contribute to this challenge. Furthermore, ART would be nearly impossible if animals from different locations or far from laboratory facilities are used. Although ART have not yet been developed for most species of nondomestic mammals, improvements in cryopreservation techniques will subsequently enhance long-term storage of biomaterials that can potentially be used in future efforts at preservation of endangered species.

##### **2.1.1 Gametes and Embryos**

The use of low temperature to preserve gametes and embryos has been a topic of interest for centuries. One such example is a proposal made in 1866 by an Italian military physician, Mantegazza, who noticed that human spermatozoa became immotile when cooled in snow and suggested that it might be possible for a soldier killed in battle to father a child after his death if fertility of spermatozoa could be maintained by cooling [cited by (Smith, 1961a)].

Almost a century after Mantegazza's proposal, Hammond suggested that if animal spermatozoa could be cooled to temperatures low enough to reduce metabolism while maintaining fertilizing capabilities, then "in these days of rapid aeroplane transport, it might be possible to move entire herds of animals around the world in form of chilled samples of semen" (Hammond, 1930).

Although some earlier reports had claimed variable success in freezing spermatozoa, it is the landmark discovery of the cryoprotective properties of glycerol by Christopher Polge, Audrey Smith and Allen Parks in 1949 that marked the beginning of the era of successful cryopreservation of living sperm cells (Polge et al., 1949). At the time, AI of cattle using fresh or cooled semen was a well established technique for producing genetically superior animals, so the newly acquired ability to cryopreserve spermatozoa provided the impetus for even greater extension of the most desirable genes. Two years after the initial discovery, the birth of the first normal live bull calf from AI using frozen-thawed spermatozoa was reported (Stewart, 1951). Since then, offspring using this procedure have been reported for most domestic and more than 40 nondomestic mammalian species. Today, cryopreservation of semen continues to have an enormous impact on commercial livestock production and is a key component of biological resource banks for endangered species conservation.

Successful oocyte cryopreservation is more difficult than is the freezing of spermatozoa (Van der Elst, 2003). The oocyte is the largest mammalian body cell and has a low surface-to-volume-ratio in comparison to other smaller cell types. Therefore, equilibration of cryopreservation medium with the ooplasm as well as prevention of internal ice crystal formation are more difficult to achieve. The acellular membrane surrounding the oocyte, the zona pellucida, may undergo physical changes during the freezing procedure that result in a modification referred to as 'hardening' (Carroll et al., 1990; Johnson, 1989; Vincent et al., 1990).

The ooplasm and zona pellucida are surrounded by layers of granulosa cells with projections that traverse the zona pellucida to form gap junctions with the ooplasm. Thus, the oocyte-cumulus complex consists of two different types of cells that must both remain functional to ensure proper maturation of the oocyte. Furthermore, the meiotic spindle, which is essential for normal fertilization and cleavage, is sensitive to cryoprotective agents as well as the freeze-thaw cycle (Pickering and Johnson, 1987; Van der Elst et al., 1988). The complex spindle structure also changes during oocyte development and maturation. Therefore, oocytes at

different stages of maturation require different protocols. For example, the spindle of immature oocytes is less sensitive to temperature and appears to be more resistant to cryo-stress than is that of mature oocytes (Agca, 2000).

Despite these challenges, live births have been produced after transfer of embryos produced from cryopreserved oocytes in the mouse (Whittingham, 1977), human (Porcu et al., 1997; Tucker et al., 1998), cow (Vajta et al., 1998; Hamano et al., 1992) and horse (Maclellan et al., 2002). No births have been reported in nondomestic species and much additional research is needed before oocyte cryopreservation will make an impact in conservation.

In contrast, a variety of methods have been used for successful cryopreservation of embryos. Since the birth of the first mouse pups from cryopreserved embryos (Whittingham et al., 1972), live births from transfer of frozen embryos have been produced in most domestic and some nondomestic species (Table 2.1).

### **2.1.2 Cell Lines and Tissues**

Shortly after the discovery of the cryoprotective properties of glycerol, the first successful cryopreservation of red blood cells was reported (Smith, 1950). By the mid 20<sup>th</sup> Century, *in vitro* cell culture techniques were improving and tissue culture was a flourishing field of research. Therefore, the successes by Polge et al. (1949) sparked renewed interest in cell culture and tissue cryopreservation and subsequently led to the development of cryopreservation techniques for different types of cells. Furthermore, freezing of tissue before *in vitro* cell culture became of interest for grafting purposes (Collier et al., 1987; Almodin et al., 2004) and for reducing or re-distributing the work load in tissue culture laboratories (Taylor et al., 1978; Gray et al., 1995). In the last few years, as more animals were produced by nuclear transfer, cryopreservation of cells and tissues became an important part of biological resource banks for supplying diploid genomes that can potentially result in cloned offspring.

Various other biomaterials, such as blood, hair, fecal and urine samples are also frequently included in biological resource banks for an array of endocrine and genetic studies of

Table 2.1. Nondomestic species in which live offspring was born following embryo cryopreservation and transfer.

Species	Date	Reference
Baboon	1984	Pope et al. (1984)
Common eland	1984	Dresser et al. (1984)
Cynomolgus monkey	1986	Balmaceda et al. (1986)
Marmoset monkey	1986	Hearn and Summers (1986)
Scimitar Horned oryx*	1986	Schmidt (1986)
Rhesus monkey	1989	Wolf et al. (1989)
Macaque**	1990	Cranfield et al. (1990)
Red deer	1991	Dixon et al. (1991)
Swamp buffalo	1993	Kasiraj et al. (1993)
Ocelot	1997	Swanson (2001)
African wildcat	2000	Pope et al. (2000)
Caracal	2003	Pope et al. (2006b)
European polecat	2003	Lindeberg et al. (2003)

\* Calf was stillborn.

\*\* Pig-tailed macaque oocyte fertilized with Lion-tailed macaque spermatozoa.

captive and wild populations. However, gametes, embryos, cells and tissues are the only bio-materials that can be used to incorporate new genes into an endangered population and can potentially have a significant impact on conservation of endangered species.

## **2.2 *In Vitro* Production of Embryos in Nondomestic Species**

The first successful embryo transfer was reported by Sir Walter Heape more than a century ago (Heape, 1890) and opened an entirely new field in reproductive biology. The ability to transfer an embryo into a surrogate mother and obtain a healthy offspring enabled scientists to produce more offspring from a specific, genetically valuable animal than what would be possible through natural breeding. In a few species, a surrogate mother from a different breed or species has been used to produce live births (Dresser et al., 1985; Lanza et al., 2000; Loi et al., 2001; Gómez et al., 2004). Therefore, the production of embryos *in vitro* has evolved from simply being a valuable research tool to a field that is having a practical impact in domestic animal production and have the potential to have a similar impact in conservation of endangered species.

### **2.2.1 Oocyte Collection**

Oocytes matured *in vivo* and *in vitro* have been used for production of embryos in several domestic species, but the use of *in vitro* matured oocytes for exotic species has been limited. Ovaries of domestic animals obtained from abattoirs and veterinary clinics are a good source of oocytes for *in vitro* embryo production. Oocytes are collected from ovaries by needle aspiration of follicles, slicing, follicle dissection and enzymatic digestion (Gordon, 1994a). The frequency at which domestic animal oocytes mature *in vitro* varies according to rigidity of selection criteria, species and culture environment. Thus, even though most good quality oocytes are competent to undergo nuclear maturation *in vitro*, the quality of embryos derived from *in vitro* matured oocytes has been inferior to those produced from oocytes matured *in vivo*. Collection of *in vivo* matured oocytes from nondomestic felid species is usually accomplished by

laparoscopy after treatment with exogenous gonadotropic hormones to enhance follicular development. Examples include lions (Armstrong et al., 2004; Damiani et al., 2004), tigers (Crichton et al., 2003), caracals and fishing cats (Pope et al., 2006b). Although, felid oocytes matured *in vivo* can be repetitively collected from individual donors by laparoscopy (Pope et al., 2006b), the process is significantly more expensive and labor intensive than the production of oocytes matured *in vitro*.

Transvaginal ultrasound-guided oocyte aspiration (TUGA) is another technique for collecting oocytes directly from the animal, which does not involve surgery and is generally considered as less invasive than laparoscopy (Pieterse et al., 1991), although it may be more problematic in smaller species like felids (C.E. Pope, personal communication). Successful TUGA procedures have been reported in various exotic species, including gaur (Armstrong et al., 1995), African buffalo, Sable antelope, tsessebe, wapiti and Red hartebeest (Loskutoff, 1995), Burchell's and Hartmann's zebra (Meintjies et al., 1997), gorilla (Pope et al., 1997; Loskutoff et al., 2004), addax (Asa et al., 1998), bongo (Pope et al., 1998b), llama (Brogliatti et al., 2000), baboon (Sceh et al., 2001), Common eland (Wirtu et al., 2002a), Red deer (Berg and Asher, 2003), Thai Swamp buffalo (Techakumphu et al., 2004) and Murrah buffalo (Gupta et al., 2005).

### **2.2.2 Semen Collection**

As with collection of oocytes, spermatozoa can be obtained either from living animals or immediately after death. Spermatozoa can be obtained from testes of domestic animals at slaughterhouses and veterinary clinics. The most likely sources for testes of nondomestic animals are game ranches or zoological institutions. Spermatozoa are harvested by mincing, rinsing or aspiration of particular portions of the epididymis and vas deferens. For live nondomestic animals, various techniques have been used to collect semen, including an artificial vagina (Skidmore et al., 2001), masturbation or auto masturbation (Brown and Loskutoff, 1998), electroejaculation (Okano et al., 2004), transrectal massage of the ampulla



and accessory glands (Wirtu et al., 2002b), from the vagina after mating (O'Brien and Roth, 2000), via a penile urethral fistula or by percutaneous epididymal sperm aspiration (PESA) (Damiani et al., 2004). The sperm cells can be used immediately after collection or cryopreserved for future use.

### **2.2.3 *In Vitro* Fertilization**

The first successful IVF was demonstrated by M.C. Chang (1959), after the simultaneous but independent discovery of sperm capacitation by Chang (1959) and Austin (1951). Since Chang's initial success in rabbits, offspring have been produced by IVF in numerous domestic and nondomestic species (the nondomestic species are listed in Table 2.2). Culture medium, temperature, oocyte quality, sperm source and time of sperm-oocyte interaction are among innumerable factors that contribute to successful *in vitro* fertilization. Domestic animals are often used as models for the development of reliable protocols for *in vitro* production of embryos that can then be applied to related species of endangered animals.

### **2.2.4 Intracytoplasmic Sperm Injection**

Intracytoplasmic sperm injection (ICSI) is a micromanipulation technique successfully used for treating male factor infertility in humans. ICSI involves the mechanical injection of a spermatozoon directly into the cytoplasm of an oocyte. Hosoi et al. (1988) reported the first birth of live offspring following the oviductal transfer of ICSI-derived embryos into pseudopregnant rabbits. Subsequently, live births have been obtained in numerous domestic species, including cattle (Goto et al., 1990), mouse (Kimura and Yanagimachi, 1995), domestic cat (Pope et al., 1998a), sheep (Catt et al., 1996), horse (Cochran et al., 1998), rat (Dozortsev et al., 1998), pig (Martin, 2000), hamster (Yamauchi et al., 2002) and human (Palermo et al., 1992). In nondomestic species, ICSI has resulted in live births of Rhesus monkey and mastomys offspring (Chan et al., 2000; Ogonuki et al., 2003) (Table 2.2). The expense of laboratory equipment and the development of technical expertise are major limitations to use of this particular ART for application to endangered species.

Table 2.2. Exotic species in which *in vitro* produced offspring have been born.

Technique	Species	Date	Reference
IVF	Rhesus monkey	1983	Bavister et al. (1984)
	Baboon	1984	Clayton and Kuehl (1984)
	Cynomolgus monkey	1984	Balmaceda et al. (1984)
	Marmoset	1988	Lopata et al. (1988)
	Indian Desert cat	1989	Pope et al. (1989)
	Tiger	1990	Donoghue et al. (1990)
	Asian buffalo	1991	Suzuki et al. (1992)
	Armenian Red sheep	1994	Coonrod et al. (1994)
	Gaur	1994	Johnston et al. (1994)
	Gorilla	1997	Pope et al. (1997)
	Mastomys	1998	Ogonuki et al. (2003)
	African wildcat	1999	Pope et al. (2000)
	Caracal	2000	Pope et al. (2006b)
	Ocelot	2000	Swanson (2001)
	Fishing cat	2002	Pope et al. (2006b)
	Mouflon	2002	Ptak et al. (2002)
	Serval	2003	Pope et al. (2006a)
	Wapiti deer	2003	Berg et al. (2003)
	Red deer	2004	Berg et al. (2004)
ICSI	Rhesus monkey	2000	Chan et al. (2000)
	Mastomys	2002	Ogonuki et al. (2003)
SCNT	Gaur	2000	Lanza et al. (2000)
	Mouflon	2001	Loi et al. (2001)
	African wildcat	2003	Gómez et al. (2004)
	Banteng	2003	Lanza et al. [see Holden (2003)]
	Deer	2003	<a href="http://www.cnn.com/2003/TECH/science/12/22/cloned.deer.ap/index.html">http://www.cnn.com/2003/TECH/science/12/22/cloned.deer.ap/index.html</a>
	Water buffalo	2005	<a href="http://www.chinaembassy.org">http://www.chinaembassy.org</a>

### 2.2.5 Somatic Cell Nuclear Transfer

Early in the 20<sup>th</sup> Century, Hans Spemann conducted a series of experiments to test one of August Weismann's theories. According to Weismann's Germ-cell Theory, unequal nuclear division during early embryonic cleavage induces cell differentiation, but as cells progressively develop and differentiate they become permanently inactivated (Weissman, 1893). In Spemann's classic 1914 study, he demonstrated totipotency in the newt by tying a hair around a zygote to cause constriction and division into a nucleated cell and an anucleated cell. At the 16-cell stage, the hair loop was loosened and a single blastomere was allowed to pass through the constriction before the loop was again tightened, thereby cutting the embryo in half. Both the multi- and single-nucleated halves developed into whole organisms, resulting in twin newts and demonstrating totipotency of the embryonic cells (Spemann, 1938).

While Spemann (1938) is generally credited as the first to propose the "fantastical experiment" of NT, the French biologist, Yves Delage (1854-1920), proposed such an experiment and correctly predicted the outcome in 1895. A short paragraph from his publication is translated by Beetschen and Fischer (2004):

"Every nucleus, at least at the beginning of ontogenesis, is a sex cell and if, without any deterioration, the egg nucleus could be replaced by the nucleus of an ordinary embryonic cell, we should probably see this egg developing without changes".

However, it was nearly a quarter of a century after Spemann's proposal before the first hatched tadpoles were produced by transplanting blastula nuclei into enucleated eggs of *Rana pipiens* (Briggs and King, 1952). Although, they confirmed the totipotency of embryonic cell nuclei, development was not as successful when more differentiated cells were used. Subsequent studies with *Xenopus* showed that even the nuclei of differentiated intestinal cells could induce the formation of an adult male and female toad (Gurdon, 1962). The birth of a healthy lamb following transfer of an adult somatic cell into an enucleated oocyte demonstrated that differentiated cells retain their potential for totipotency (Wilmut et al., 1997), but nuclear reprogramming of a fully differentiated adult cell is more complex than reprogramming

embryonic cells that are early in the differentiation process (Kikyo and Wolffe, 2000; Tsunoda and Kato, 2002).

Since the groundbreaking discovery by the Roslin Institute (University of Edinburgh), NT has resulted in live births in several domestic mammalian species, including cattle (Cibelli et al., 1998; Kato et al., 1998), mice (Wayakama et al., 1998), goats (Baguisi et al., 1999), pigs (Polejaeva et al., 2000), cats (Shin et al., 2002), horses (Galli et al., 2003), mules (Woods et al., 2003), rats (Zhou et al., 2003) and a dog (Lee et al., 2005). The potential of nuclear transfer technology for endangered species conservation has been reviewed (Ryder and Benirschke, 1997; Comizzoli et al., 2000; Ryder, 2002; Latham, 2004; Pukazhenthi et al., 2004) and a few endangered species clones have been born (Table 2.2). The ability to clone animals from cryopreserved cell lines may be the most important motivation for the storage of cell lines in frozen zoos as a “genetic backup” of the extant population. However, much additional research and extensive improvement is needed before NT can truly make an impact on conservation.

## **2.3 Tissue Culture**

### **2.3.1 History of *In Vitro* Cell Culture**

The early history of the development of tissue culture has been described in numerous publications. The following account of the process was compiled from five book chapters (Parker, 1961; Willmer, 1965; Paul, 1972, 1975; Lee, 1990) that independently summarize the sequence of events occurring during the late 19<sup>th</sup> and early 20<sup>th</sup> Century. The early days of tissue culture are described and key events and subsequent research are listed in Table 2.3.

The term “cell culture” is explained originally “as the maintenance and growth of explanted tissue (plant or animal) in culture away from the source organism”, but now usually

Table 2.3. Selected historical events that led to modern cell culture techniques.

Date	Event	Name
1665	Small holes in cork cross sections termed “cells”.	Hooke
1692	Formulation of the so called “cell theory” which stated that all life is based on individual cells.	Schleiden and Schwann
1855	“Cell theory” improved in formulating that all cells are derived from other cells and do not form spontaneously.	Virchow
1866	Amphibian blood cells kept alive in sterile containers under various conditions for 35 days.	von Reckling-Hausen
1878	Importance of internal environment in regulating activities of living tissue.	Bernard
1885	Maintain medullary plate of chick embryo in warm saline for few days.	Roux
1891	Totipotency of embryonic cells demonstrated in the sea urchin.	Driensch
1887	Migration and short survival of leucocytes into warm saline in dish from alder pith implanted in frogs.	Arnold
1903	Division of amphibian leukocytes outside the body for 1 month.	Jolly
1906	Cultivation of infectious canine lymphosarcoma in blood from resistant and susceptible animals.	Beebe and Ewing

(table continued)

1890s	Formulation of the Germ-plasm Theory.	Weismann
1907	Observed outgrowth of frog nerve fibers into clotted lymph with hanging-drop method. Marks beginning of tissue culture.	Harrison
1910	Used techniques in warmblooded animals. Cultured chick embryo tissue in fowl plasma/serum.	Burrows; Carrel
1911	Culture chick embryo tissues in simple salt solutions supplemented with chick bouillon.	Lewis and Lewis
1913	Tissue culture becomes “headline news” with Carrel’s publication on artificial activation of growth and cell division by means of saline extracts from embryonic tissues.	Carrel
1914	Presence of mitochondria in cells in tissue culture demonstrated by vital staining.	Lewis and Lewis
	Demonstration of embryonic cell totipotency in vertebrate (newt).	Spemann
1922	Differentiation of epithelial cells cultured in colonies with fibroblasts observed.	Chlopin; Ebeling and Fischer
1922	One of the earliest and most complete descriptions of the process of cell division.	Strangeways
1923	Development of first tissue culture flasks (Carrel flask).	Carrel
1930s	Medias recognizable as precursors for today’s media produced.	Vogelaar and Erlichman Baker
1934	Treatment of cells in hypotonic solution disperses the chromosomes throughout the cytoplasm instead of allowing them to accumulate upon the metaphase plate.	Lewis

(table continued)

1940s	Use of antibiotics in tissue culture introduced.	Pelcak
1946	Necessity for synthetic or defined medium with known composition is realized.	White
1949	Glycerol as cryoprotectant discovered. Protocol developed that allows freezing of cells in suspension and storage at in liquid nitrogen at -196°C.	Polge, Smith and Parkes
1952	Develop a reliable and practical method of dispersing viable cells from parent tissue using trypsin.	Moscona and Moscona
1955	First version of Eagle's medium published. Identified 12 essential amino acids and seven required water-soluble vitamins for cell culture.	Eagle
1960s	Significant progress towards eliminating natural supplements in cell culture.	
1962	3T3 cell line established from Swiss mouse embryos.	Todaro and Green
1975	Use of feeder layers in epithelial cell culture.	Rheinwald and Green
1978	Use of collagen as biological matrix for epithelial cell growth.	Liu and Karasek
1970s to 1980s	Discovery of cell cycle regulatory mechanisms.	Hartwell; Nurse and Hunt
1988	Purification of mitoses promoting factor (MPF).	Lohka
1996	Demonstration of totipotency of adult cells with birth of first adult cloned mammal Dolly.	Wilmut and Campbell

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(Parker, 1961; Willmer, 1965; Paul, 1972, 1975; Lee, 1990)

refers to “the technique of cell culture using cells dispersed from tissues or distant descendants of such cells” (as cited by Cancerweb online medical dictionary: <http://cancerweb.ncl.ac.uk/omd/>).

The history of cell culture goes back to the late 19<sup>th</sup> Century and since one field of study can initiate interest and lead to new, often “accidental” discoveries in another, it can probably be traced back even further than that. One such example is the discovery of trypsin for tissue culture in 1856 when Claude Bernard was seeking an explanation for earlier observations made by Hunter, who noticed that although living stomach and intestine were not digested by their own juices, such tissues were rapidly digested after death. Fermi (1910) later reported that many cells and organisms can tolerate prolonged trypsin exposure. By 1952, almost a century after Hunter’s observation, a reliable method of trypsin disaggregation for cell culture was developed by the Mosconas. To give an accurate, detailed account of the historic events that led to modern methods of cell culture would be a complicated task. Therefore, only a select group of events that are either directly related to or led to current techniques that were used in conducting the present experiments will be mentioned. Clearly, it is not an all inclusive list and not meant to imply that omitted events were of less significance in the development of tissue culture techniques.

Although several observations of cell survival *in vitro* were made in the 19<sup>th</sup> Century, in 1907, Harrison was the first to describe a successful technique for *in vitro* tissue culture. In that study, nerve tissue from the spinal cord of a tadpole was cultured in clotted lymph from the frog, and after several days of culture nerve fibers outgrowth was observed. These experiments were performed by the hanging-drop method where a drop of medium is placed on a coverslip which is then inverted over a hollow-ground microscope slide and sealed with paraffin wax (Harrison, 1907), a method that is still sometimes used today. Burrows, a student studying under Harrison’s direction (at the time at the Rockefeller Institute in New York), applied the same technique of culturing cells from warmblooded animals and discovered the use of animal serum



as a culture medium (Burrows, 1910). In collaboration with Ebeling and Alexis Carrel (a highly skilled surgeon), the foundation was laid for continuous culture of a variety of tissues (Willmer, 1965). Carrel's surgical experience led to the development of many of the basic aseptic techniques used in cell culture. He also emphasized the importance of interaction between cells and the surrounding medium in a dynamic system (Carrel, 1913).

The original findings by Harrison (1907) also initiated interest in cell culture by Warren and Margaret Lewis in Baltimore, who investigated the factors needed in the culture medium for cell survival and growth and the importance of controlling and defining the cellular environment [see (Waymouth, 1972)]. Such studies were directly leading to cell culture methods that permitted rapid cell growth; however, David Thompson (1914), and then Strangeways and Fell (in England) were more concerned with developing techniques for maintaining the *in vivo* integrity of tissue fragments in an *in vitro* environment (i.e., organ culture).

After World War I (1914-1918), international interest in tissue, organ and cell culture expanded rapidly and groups in countries such as Denmark, Germany, France, Russia and Italy became actively involved. Although some success was obtained, the continuous battle against microbial contamination and the tedious methods associated with the aseptic technique largely developed by Carrel, discouraged many biologists. Overall, the general belief was that tissue culture was extremely difficult and, as a result, progress in cell culture slowed tremendously. The introduction of antibiotics in cell culture in the mid 1940s renewed interest and led to an enormous expansion of the field.

Today, almost a century after those challenging early days, cell culture has developed into a powerful technique that significantly contributes to every field of experimental biology and medicine. Major advances in areas such as cytology, histology, embryology, cell physiology, cell pathology, bacteriology and immunology and in the study of tumors and viruses can be attributed to development of and improvement in tissue culture systems.

In reproductive biology, cell culture continues to play a variety of important roles, such as in co-culture systems for animal (Goto et al., 1988; Eyestone and First, 1989; Carney et al., 1990; Ouhibi et al., 1990; Prichard et al., 1992; Smith et al., 1992; Yan et al., 2000; Locatelli et al., 2005) and human (Wiemer et al., 1989; Thibodeaux and Godke, 1995) embryos, in the production of conditioned culture media (Eyestone et al., 1989; Li et al., 2004) and karyoplast donors for nuclear transfer procedures (Wilmut et al., 1997; Comizzoli et al., 2000; Wolf et al., 2001; Brem and Kuhholzer, 2002) and, more recently, in the field of embryonic stem cell culture (Downing and Battey, 2004; Draper et al., 2004; Gerecht-Nir and Itskovitz-Eldor, 2004; Gilbert, 2004; Shufaro and Reubinoff, 2004).

### **2.3.2 The Cell Environment**

In 1913, Alexis Carrel described the significance of the cell environment and interactions between the cell and its immediate surroundings on survival and proliferation *in vitro* (Willmer, 1965). The cell is dependant upon its immediate environment for energy sources and for the exchange of substances required for maintaining structure and function. However, the surrounding environment must remain constantly favorable for optimal survival since cells are affected immediately if the milieu is negatively altered. Key environmental components include temperature, pressure, composition (a function of water and its solutes) and pH, all of which are independent of volume or quantity of milieu provided to the cells in culture (Ogston, 1973). All of these properties must be optimized to allow the complex inter- and extracellular biochemical activities necessary to sustain normal metabolism. Therefore, it is of utmost importance to ensure that the incubation temperature, medium salt concentrations, buffering system and humidity are maintained favorable and monitored closely during *in vitro* cell culture.

### **2.3.3 Nutritional Requirements of Cells in Culture**

Nutritional requirements for *in vitro* cell metabolism and proliferation are similar to that of *in vivo* cells. Amino acids are among the most important of the nutritional factors since they are (a) building blocks of proteins, (b) indispensable agents of biological structure, (c) function

as enzymes and hormones, (d) components of transport systems, (e) components of translation and gene expression, (f) mediate cell movement and (g) serve as antibodies (Garret and Grisham, 1995a; Horton et al., 1996). Of the thousands of proteins found in living organisms, all are comprised of different combinations of 20 amino acids (Garret et al., 1995a).

Vitamins are small molecular weight compounds, some of which are water-soluble (like B-vitamins and Vitamin C), and some are fat soluble (like Vitamins A, D and E). Water-soluble vitamins (except for Vitamin C, which is a strong reducing agent) are components or precursors of co-enzymes, which are mandatory for specific enzyme reactions. Fat soluble vitamins play essential roles in critical biological processes like maintenance of bone structure, coagulation of blood and acuity of vision (Garret and Grisham, 1995b).

Cells require a constant energy source to drive metabolic processes. Although carbohydrates are the key energy additives in culture media, cells can also utilize other carbon compounds, such as keto acids, carboxylic acids, purines and pyrimidines. D-glucose is the major sugar in mammalian serum and is therefore included in most culture media. However, some catabolic products of glycolysis and the citric acid cycle, for instance pyruvate, lactate and glutamate can be metabolized through alternative energy pathways and are also suitable energy sources for *in vitro* cell culture (Waymouth, 1972). Recent findings suggest that the citric acid cycle may not function *in vitro* as *in vivo*, and many cell types require glutamine or glutamate (Freshney, 1983b). Most modern media thus contain a combination of different types of energy substrates that can be utilized by numerous cellular processes.

Therefore, it is mandatory that *in vitro* culture media supply the necessary amino acids, vitamins and energy substrates at the appropriate levels needed for cellular function and metabolism.

In a classic study, Harry Eagle used a basal medium composed of 19 amino acids, 15 vitamins, inorganic salts and additional growth factors for culturing mouse fibroblast L cells (Eagle, 1955f). By omitting one component at a time while monitoring cell proliferation, he was

able to identify 12 amino acids essential for the growth of L cells (Table 2.4). Although, glutamine was determined not to be essential for proliferation, he continued adding glutamine as an additional factor. Dr. Eagle proceeded to determine the effective concentration of each essential amino acid and then repeated the experiment with HeLa cells (derived from a human carcinoma in 1951; (Eagle, 1955e), as well as many other cell lines (Eagle et al., 1957; Eagle et al., 1958b). He also performed similar experiments by omitting vitamins from the basal medium to identify essential vitamins and their optimal concentrations (Eagle, 1955d). No fat-soluble vitamins were found to be essential and seven water-soluble vitamins were essential for L cells and HeLa cells (Table 2.4). Requirements of amino acids and vitamins were identical for L cells, although optimal concentrations differed between cell lines.

Inorganic salt requirements of culture media were defined in other studies (Eagle, 1956). Additionally, the role of glutamine (Eagle et al., 1956; Levintow et al., 1957; Darnell and Eagle, 1958; Salzman et al., 1958), carbohydrates (Darnell et al., 1958; Eagle et al., 1958a), antibiotics (Eagle et al., 1955; Eagle, 1955c; Eagle and Zak, 1955) and phenols (Oyama and Eagle, 1956) on cell growth have now been defined.

All of the previously mentioned studies contributed to the development of Eagle's minimum essential medium (MEM) for cultivation of mammalian cells in either monolayer or suspension (Eagle, 1959) (Table 2.5). Various modifications of the original medium are available. The enormous impact that this first "defined" medium had on tissue culture research and especially formulation of different media is still apparent. In fact, 16 years after publication of the original MEM medium, different Eagle's media were used in >60% of all tissue culture studies that appeared in leading journals (Yamada, 1979).

### **2.3.4 Culture of Specific Cell Types**

Cell types are classified according to their behavior after being allowed to grow from a piece of tissue onto a flat surface (Willmer, 1960). Examples of different cell types include epitheliocytes, mechanocytes (fibroblasts), amoebocytes, nerve cells, neuroglia cells and

Table 2.4. Essential amino acids and vitamins for mammalian *in vitro* cell culture.

Amino Acids	Vitamins
Arginine	Biotin
Cystine	Choline
Histidine	Folic acid
Isoleucine	Nicotinamide
Leucine	Pantothenic acid
Lysine	Pyridoxal
Methionine	Thiamine
Phenylalanine	Riboflavin
Threonine	
Tryptophan	
Tyrosine	
Valine	

(Eagle, 1955f)

Table 2.5. Eagle's minimum essential medium (MEM) for cultivation of mammalian cells in either monolayer or suspension.

Compound	Concentration		Compound	Concentration	
	mM	mg/L		mM	mg/L
L-amino acids			Salts		
Arginine	0.6	105	NaCl	116	6,800
Cystine	0.1	24	KCl	5.4	400
Glutamine	2.0	292	CaCl <sub>2</sub>	1.8 (0) <sup>a</sup>	200
Histidine	0.2	31	MgCl <sub>2</sub> •6H <sub>3</sub> O	1.0	150 (1,500) <sup>a</sup>
Isoleucine	0.4	52	NaH <sub>2</sub> PO <sub>4</sub> •2H <sub>2</sub> O	1.1	2,000 <sup>a</sup>
Leucine	0.4	52	NaHCO <sub>3</sub>	23.8 (11) <sup>a</sup>	
Lysine	0.4	58	Vitamins		
Methionine	0.1	15	Choline		1
Phenylalanine	0.2	32	Folic acid		1
Threonine	0.4	48	Inositol		2
Tryptophan	0.05	10	Nicotinamide		1
Tyrosine	0.2	36	Pantothenate		1
Valine	0.4	46	Pyroxidal		1
			Riboflavin		0.1
Carbohydrate			Thiamine		1
Glucose	5.5	1,000	Serum protein		
			Whole or dialyzed serum (5 to 10%)		

(Eagle, 1959)

<sup>a</sup> In suspension cultures.

lymphocytes. More recently, the cell type classification has been based on their embryonic origin (epithelial, mesenchymal, neurectodermal and hemopoietic cells), with anatomical subgroups (Freshney, 1983a). In some studies the organ from which cells were derived served as the basis for distinguishing cell types and the specific tissue layer from which the cells originated was not considered. For example, Kato et al (2000) compared the developmental potential of NT embryos derived from “different cell types”, but they did not report the specific cell types that were being used. Although the donor tissues were derived from such organs as skin, ear, liver and lung, cells from each of the organs were, quite possibly, all fibroblast cells and not truly different cell types, especially since the same culture medium was used for all of the cell lines.

Even though cells are sometimes characterized morphologically, it is an inadequate criterion since environment has an enormous influence on cell behavior. Furthermore, some cell types, such as endothelial and epithelial cells, are quite similar in appearance (Lee, 1990). A more reliable method for cell type classification consists of immunohistochemical detection of intermediate filament proteins. Specific intermediate filament proteins are characteristic of particular cell types. Examples include cytokeratins in epithelial cells, vimentin in mesenchymal cells and desmin in myogenic cells (Steinert and Roop, 1988). Each cell type requires a different culture medium and system for optimal growth.

Although a basic cell culture medium may support some proliferation of several cell types, specific requirements of every aspect of the culture system, from the nutrients and supplements in the medium to the growth surface and temperature, exist for several cell types and sub-types (Freshney, 1983a; Freshney and Freshney, 2002). The culture system may also vary for a single cell type depending on the differentiation status or functional purpose. Murine mammary epithelial cells, for example, will only synthesize milk proteins (e.g., casein) if they are cultured in the presence of insulin, hydrocortisone and prolactin. In contrast, when mammary gland cells from virgin mice were exposed to the same hormones, the cells failed to produce

casein (Juergens et al., 1965). These results indicate that there were differences in the degree of cell differentiation within the same cell type.

#### **2.3.4.1 Fibroblast Cell Culture**

Fibroblast cells were used to develop most of the early methods in cell culture and their cultivation is generally considered to be less complex than the cultivation of other cell types (e.g., epithelial or endothelial cells) (Freshney and Freshney, 2002). Also, fibroblast cells have been extensively used as feeder layers for culturing other types of somatic cells (Macpherson and Bryden, 1971; Taylor-Papadimitriou et al., 1977; Malick and Langenbach, 1979; Rabinovitch et al., 1979; Rheinwald, 1980; Tuan et al., 1994) and embryos (Wetzels et al., 1992; Kuzan and Wright, 1982). Fibroblasts have been the preferred cell type for nuclear transfer (Cibelli et al., 1998; Lanza et al., 2000; Galli et al., 2003; Woods et al., 2003; Gómez et al., 2004; Sansinena et al., 2005) transgenesis (Reggio et al., 2001; Arat et al., 2002; Iguma et al., 2005) and biological resource banking (personal communication and experience at the Audubon Nature Institute Center for Research of Endangered Species BRB). Furthermore, fibroblast cell proliferation does not require special media or substrate and basal media such as those developed by Eagle are generally adequate (Eagle, 1955a, 1955b).

#### **2.3.4.2 Epithelial Cell Culture**

##### **2.3.4.2.1 Origin, Histology and Functions of Epithelium *In Vivo***

Epithelium is “the covering of internal and external surfaces of the body, including the lining of vessels and other small cavities. It consists of cells joined by small amounts of cementing substances” (as cited by Cancerweb online medical dictionary: <http://cancerweb.ncl.ac.uk/omd/>). With the exception of the collecting duct lining of the kidney and the mesothelium that lines body cavities, all epithelial cells originate from the ectoderm and endoderm of the mammalian embryo (Freshney, 2000; Gilbert, 2000).

Epithelial monolayers separate the luminal compartment of transport tubes from the interstitial fluid, thus protecting the “inside” environment from the “outside” environment.



Therefore, the differentiated function of such epithelial monolayers is to transport fluids, such as secretions from glands, digestive fluids and blood from one compartment to another, while acting as a barrier against fluids potentially contaminated with microorganisms (Hirst, 1990).

Although epithelial cells are present in many tissues in the body and possess diverse functions, all of them share some common characteristics, including morphological polarity, interconnection by tight junctions and the capacity for vectorial transport of pollutants across the monolayer. Dome formation is an indicator of this vectorial transport function in many types of epithelial cells *in vitro* (Sugahara et al., 1984).

Because of their functional purposes, the top layers of epithelial cells are constantly being sloughed off, so the population requires constant renewal. Accordingly, most epithelial cells are renewable with proliferating precursor compartments and stem cells capable of self-renewal. Regeneration occurs at different rates among various organs according to the extent of exposure to sloughing factors (i.e., rapid regeneration of epidermal cells). Stem cells located in specific regions of organs constantly replenish the functional portion of epithelium as the terminally differentiated cells reach senescence, die and are shed or sloughed off (Freshney, 2002).

#### 2.3.4.2.2 *In Vitro* Culture of Epithelial Cells

Since epithelia are associated with the major functional roles of various tissues and vital organs, *in vitro* models and proliferation of epithelial cells have been a major focus of biomedical research (Freshney, 2000). This is especially relevant for cancer research since most malignant tumors are of epithelial origin. In general, epithelial cell culture is considered to be a more specialized technique than fibroblast cell culture. Culture systems that mimic the *in vivo* environment have evolved for various types of epithelia, with specialized modifications to all aspects of the culture system, including the initial disaggregation of the primary cells, growth surface properties, medium composition, incubation temperature and additives to control differentiation, all of which contribute to optimal proliferation [for review, see (Freshney and

Freshney, 2002)]. However, many of the modifications are aimed at preventing overgrowth of the culture by fibroblast cells and are not necessarily a requirement for epithelial growth (Freshney, 1983a).

Epithelial cell culture could be of potential value for conservation purposes, especially if fibroblast cells are not available. Since epithelial cells are present in almost all tissues, establishing such cells in culture concurrent with fibroblast cells from the same donor animal would maximize the opportunity that those genes would be available for future use. Epithelial cells are also present in bodily fluids that may not contain fibroblast cells and their isolation is under investigation. Cells from bodily fluids potentially can serve as a noninvasive source of cells for culture and genomic resource banking.

## **2.4 The Gulf Coast Native Sheep and Eland Antelope as Model Species**

The Gulf Coast Native (GCN), also known as the Louisiana Native, is a domestic breed of sheep that arrived in the United States from Europe in the 16<sup>th</sup> Century. The transplanted population endured centuries of natural selection in the warm, humid environment of the Gulf Coast region and developed unique characteristics that made them valuable for modern day sheep breeders (Mason, 1988). One of their most sought after characteristics is their natural resistance to parasites, especially gastrointestinal nematode parasites, such as *Haemonchus contortus* (Miller et al., 1998; Li et al., 2001), as well as foot rot (Whittington and Nicholls, 1995). There is a special interest in this breed for use in crossbreeding programs in an effort to introduce their valuable characteristics to other wool and hair sheep breeds (Li et al., 2001). Since the status of GCN breed is listed as “critical” under the American Livestock Breeds Conservancy, it is important to manage the extant population such that maintenance of genetic diversity is ensured. Banking of biological materials from this breed will assist breeders in managing and protecting the small residual population.

The current research was performed in Louisiana, where extreme summer temperatures and humidity place an enormous amount of stress on domestic farm animals. Adding additional stressors, such as semen collection or biopsy procedures, are potential health risks to more sensitive breeds. The Louisiana State University Agricultural Center Sheep Research Unit is part of a breeding and research program for the GCN and has a flock with rams of known fertility. Therefore, considering the availability and hardiness of these animals, the GCN sheep is an excellent choice as a model species for developing procedures that may benefit endangered species conservation. The Common eland (*Taurotragus oryx*) is the second largest African antelope and one of nine spiral-horned antelope species of the tribe Tragelaphini. Two genera exist, namely *Tragelaphus* (bongo, lesser and greater kudu, sitatunga, nyala, Mountain nyala and bushbuck) and *Taurotragus* (Common and Giant eland), although eland has been placed in the *Tragelaphus* genus based on molecular data (Essop et al., 1997). Three of the 18 subspecies that make up the two genera are endangered, while at least one *Taurotragus* species is thought to be extinct.

Although the Common eland is not considered endangered, ART research in this species could be of value should the knowledge be extrapolated to other endangered relatives. The birth of a healthy, endangered bongo calve to an eland antelope following interspecies ET demonstrated that one species could be used in ART of another (Dresser et al., 1985). Several reports assisted reproduction techniques in Tragelaphini have been published and since the Common eland is among the most studied, it is an appropriate model species for other Tragelaphini [see Tables 2.1, 2.2 and (Wirtu, 2004)].

The Audubon Research Center has a herd of eland that is conditioned to voluntarily enter a handling chute for oocyte and semen collection procedures (Wirtu, 2004). Furthermore, successful gamete collection techniques were established in the male and females. Therefore, the animals were used for extrapolating our studies in sheep to this species.

## CHAPTER 3

### **FREEZING BIOPSIES UNDER FIELD CONDITIONS: COMPARISON OF DIFFERENT FREEZING MEDIA AND EQUILIBRATION TIME ON CELL SURVIVAL IN DERMAL AND EPIDERMAL LAYERS OF FLASH-FROZEN BIOPSIES**

#### **3.1 Introduction**

*In vitro* culture of mammalian cells is widely used in innumerable scientific studies. The technology has become a valuable tool for many specialists from basic cell biologists to bioengineers and pharmaceutical manufacturers (Freshney, 2000). Cell culture has also become an important aspect for conservationists in the form of biological resource banking in which tissue and cell lines of endangered species are frozen and used in a wide array of studies, such as phylogeny and disease research (Wildt et al., 1997, 2000; Holt, 2001). Furthermore, if stored properly, cell lines can potentially be used for somatic cell nuclear transfer (SCNT) cloning, during which cultured cells from domestic (Wilmut et al., 1997; Cibelli et al., 1998; Kato et al., 1998; Wayakama et al., 1998; Baguisi et al., 1999; Polejaeva et al., 2000; Shin et al., 2002; Galli et al., 2003; Woods et al., 2003; Zhou et al., 2003; Lee et al., 2005) or exotic (Lanza et al., 2000; Holt, 2001; Loi et al., 2001; Gómez et al., 2004; Janssen et al., 2004; Sansinena et al., 2005) species are used as donor karyoplasts for transfer into enucleated oocytes, which may result in embryos and offspring after transfer into recipient females.

Obtaining fresh tissue samples and establishing cell lines under normal laboratory conditions is fairly simple, but under field conditions the lack of equipment and of fresh biological media can create difficulties with collecting and culturing tissue samples and maintaining a sterile environment for the cells in culture. Therefore, the banking of cryppreserved tissue prior to culture could have practical advantages. Cell lines have been successfully derived from frozen-thawed tissues using various methods (Taylor et al., 1978; Smith and Richas, 1993; Gray

et al., 1995; Sommer et al., 1999; Negishi et al., 2002; Silvestre et al., 2002, 2003, 2004).

Different freezing media, equilibration times, tissue types, cooling rates, storage times and storage temperatures are among the factors that have been evaluated. These studies found no or minor differences between cell lines derived from fresh or frozen epithelial, connective or brain tissues when assayed by different criteria. These included growth patterns, chromosomes and enzyme activity (Taylor et al., 1978), karyotype (Smith et al., 1993), the time required for the first cells to plate and reach confluence on the culture dish (Silvestre et al., 2004), success in deriving cell lines (Gray et al., 1995) and the ratio of cell types obtained from tissue (Sommer et al., 1999). Furthermore, Fahrudin et al. (2001) reported no significant difference between developmental competence of embryos derived from nuclear transfer with cell lines derived from fresh or frozen tissue. Six cow pregnancies were obtained, but no live births resulted.

In the present study, we examined the effect of different media, equilibration times and tissue types on the success of deriving cell lines from flash-frozen skin biopsies, with the specific objective to develop a practical method for use during field excursions by conservation biologists.

## **3.2 Materials and Methods**

### **▪ Experimental design**

There were four medium treatment groups: (1) blood, (2) polyvinyl pyrrolidone, (3) fetal bovine serum and (4) fresh control (Table 3.1). For each treatment, 0 minutes, 15 minutes and 30 minutes of equilibration time periods were used. Then, each biopsy was divided into dermis and epidermis tissue for separate culture and evaluation.

The data collected were the number of days until (a) cells were observed, (b) primary confluence was reached and (c) first-passage confluence was reached in each culture. A total of four replicates (no. of animals) were performed and 10 biopsies were collected from each animal to produce a total of 40 biopsies and 80 cell cultures.

Table 3.1. Medium treatment groups that were used to flash-freeze GCN x Suffolk crossbred sheep skin biopsies in liquid nitrogen in the field, following 0, 15 or 30 minutes of equilibration.

Treatment	Description
Blood	Animal's own, fresh blood + 10% dimethylsulfoxide (DMSO)
FBS	Ca <sup>2+</sup> and Mg <sup>2+</sup> -free Dulbecco's phosphate-buffered saline (DPBS) + 10% fetal bovine serum (FBS) + 10% DMSO
PVP	DPBS + 6 mg/L polyvinyl pyrrolidone (PVP) (Mol. Wt. 10,000) + 10% DMSO
Fresh	Biopsies collected and stored fresh in PBS until processing

- **Animals**

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University (LSU). The sheep were housed at the LSU Agricultural Center Sheep Research Unit in Baton Rouge, Louisiana, where the collection procedures were performed.

- **Chemical reagents**

All chemicals were obtained from Gibco (Grand Island, NY, USA) unless stated otherwise.

- **Cryopreservation media and vials**

Plastic cryogenic vials (1 mL) (Nalgene, Rochester, NY) were used to freeze single biopsies in liquid nitrogen in the field. The night before sample collection, labeled vials were prepared in the lab. For Treatment (TRT) 1, cryovials remained empty, but glass blood collection tubes were filled with 500  $\mu$ L (10% v/v of medium) of dimethyl sulfoxide (DMSO, Sigma Chemical Company, St. Louis, MO, USA) by use of a sterile syringe and needle. For TRT 2 to 4, a total volume of 1 mL medium was aliquoted into each vial. Medium for TRT 2 consisted of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free Dulbecco's phosphate-buffered saline (DPBS), 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) and 10% DMSO. For TRT 3, medium was identical to that used in TRT 2 except that the FBS was replaced by 6 mg/mL of polyvinyl pyrrolidone (PVP, Mol. Wt. =10,000, Sigma Chemical Company), as a nonbiological macromolecule. TRT 4 (fresh control) vials contained PBS supplemented with 1,000 IU/mL of penicillin and 1 mg/mL streptomycin. The medium-containing tubes were stored in the refrigerator at 5°C until usage the next day.

- **Collection and cryopreservation of tissue samples**

Gulf Coast Native (GCN) x Suffolk ewes (n=4) (Figure 3.1) were used as tissue donors. A 6-mm diameter disposable biopsy punch was used to collect 10 skin biopsies from each animal. The tissue samples were obtained from the soft skin on the medial surface of the rear leg



Figure 3.1. One of four Gulf Coast Native x Suffolk crossbred ewes from which skin biopsies were collected.



after shaving and sterilizing the skin. Immediately after collection, each biopsy was placed in a cryogenic vial containing 1 mL of the appropriate medium for each treatment.

Treatment groups are summarized in Table 3.1. For TRT1, fresh blood was obtained from the tissue donor prior to tissue collection using a syringe and needle. A 4.5 mL fraction was then added to the glass tube containing 500  $\mu$ L of DMSO to make up a final concentration of 10% DMSO in blood. After gently mixing the tube contents, 1 mL aliquots were placed in each cryogenic vial of TRT 1. The temperature of the vials containing medium for TRT 2 and 3, adjusted to the outside temperature at the time of biopsy collection ( $\sim 27^{\circ}\text{C}$ ). One biopsy was placed in each vial of each treatment. For each group, biopsies were allowed to equilibrate for 0, 15 or 30 minutes in the vial before it was submerged into liquid nitrogen ( $\text{LN}_2$ ). For TRT 4, the vials were not frozen, but transported to the laboratory fresh for processing. After a minimum of 7 days, the cryopreserved biopsies were thawed by placing the vials in a water bath at  $37^{\circ}\text{C}$  until the entire volume of medium had melted. The biopsies were washed in DPBS supplemented with 1000 IU of penicillin, 1 mg/mL of streptomycin and 2  $\mu\text{L/mL}$  gentamicin for 30 minutes. Each sample was then processed for *in vitro* cell culture.

Biopsy processing was performed by visually separating the dermis and epidermis using a heat-sterilized forceps, scalpel and blade. The tissues were then minced with sterilized dissecting scissors in DPBS and then cultured separately in 35-mm tissue culture plates (Corning, Sigma Chemical Company). The culture medium (CM) consisted of Dulbecco's Modified Eagle Medium (DMEM, see Appendix B1) containing 15% FBS, 10  $\mu\text{L/mL}$  MEM nonessential amino acid solution (Sigma Chemical Company), 100 IU of penicillin and 0.1 mg/mL of streptomycin. The primary culture was incubated at  $37.5^{\circ}\text{C}$  in a humidified atmosphere of 10%  $\text{CO}_2$  and 90% air until the cells reached 75% confluence. Subpassage was performed at this stage by dissociating the cells by PBS incubation and passing the suspension into a 75-mm<sup>2</sup> tissue culture flask. When confluence in the flask reached 90% (Passage 1

confluence), cells were dissociated with 0.25% trypsin in DPBS and cryopreserved in CM supplemented with 10% DMSO. The number of days that was required for plated cells to be observed in primary culture, as well as the time for confluence to be reached in primary and the first passage cultures were recorded throughout the experiment.

#### ▪ **Statistical analysis**

All statistical calculations were performed using the Statistical Analysis System package (SAS Institute Inc., Cary, NC, USA) and the data are presented as means $\pm$ SE, with a P-value <0.05 considered significant. To determine if there were differences between the four animals, a Kruskal-Wallis test was performed as part of the NPAR1WAY procedure. The GLM-procedure with Duncan's Multiple Range test was performed to determine relationships of the treatments within groups, while the MEANS procedure was used to calculate mean values and standard deviations within each treatment, followed by a multiple analysis of variance (MANOVA) in the GLM procedure to determine relationships between different treatment groups. Standard deviations were then used to calculate the standard error (SE) of the mean for each treatment group.

### **3.3 Results**

Results are presented in Table 3.2. No significant differences were detected among the four animals when any of the three variables were evaluated ( $P>0.05$ ). For the first variable (day on which cells were observed in primary culture), results from the GLM procedure indicated no significant differences among the medium groups ( $P=0.58$ ) and no significant difference between equilibration time groups. Significant differences were detected between tissue types ( $P=0.004$ ), with epidermis superior to dermis (Figure 3.2). Furthermore, epidermal tissue resulted initially in primarily epithelial-like colonies (Figure 3.3 A) with fibroblasts taking over the culture dish only after passage of the primary culture, whereas, connective tissue only resulted in fibroblast cells (Figure 3.3 B) from the start. Also, for the first variable (days until cells

Table 3.2. Results of cell culture from frozen-thawed sheep biopsies collected and flash-frozen under field conditions in various media and equilibration times. Data are presented as mean days $\pm$ SE.

Treatment	n <sup>a</sup>	Tissue and equilibration time					
		Dermis			Epidermis		
		0 minutes	15 minutes	30 minutes	0 minutes	15 minutes	30 minutes
Cells observed in primary culture							
Blood	4	7.75 ±2.3	5.25 ±0.48	4.00 ±0.41	4.00 ±0.71	3.25 ±0.63	3.25 ±0.25
PVP	4	7.25 ±2.59	5.33 ±0.67	5.00 ±1.00	4.75 ±1.03	3.25 ±0.25	4.25 ±0.95
FBS	4	5.00 ±0.00	5.00 ±0.00	5.00 ±0.00	5.50 ±0.50	5.00 ±0.00	5.00 ±0.00
Fresh	4	3.25 ±0.25	-	-	3.75 ±0.75	-	-
Primary culture confluence							
Blood	4	17.00 ±1.53	14.50 ±0.65	15.00 ±1.15	13.50 ±0.65	13.00 ±0.71	12.50 ±0.29
PVP	4	25.0 ±1.00	22.25 ±2.32	22.75 ±0.63	21.00 ±1.73	19.75 ±1.44	21.25 ±0.80
FBS	4	15.25 ±2.63	13.25 ±0.75	13.25 ±0.75	13.25 ±0.75	12.75 ±0.75	12.00 ±0.00
Fresh	4	11.00 ±0.00	-	-	11.00 ±0.00	-	-
Passage 1 confluence							
Blood	4	23.33 ±2.73	20.25 ±2.29	18.00 ±1.15	16.50 ±0.65	16.00 ±0.71	15.50 ±0.50
PVP	4	30.75 ±0.75	29.00 ±2.65	28.50 ±1.50	26.75 ±1.60	25.50 ±1.50	27.00 ±1.73
FBS	4	18.33 ±1.33	19.00 ±1.16	20.00 ±1.00	19.50 ±1.96	18.50 ±0.96	20.00 ±2.38
Fresh	4	15.00 ±0.00	-	-	15.00 ±0.00	-	-

<sup>a</sup> Number of animals in each treatment group.

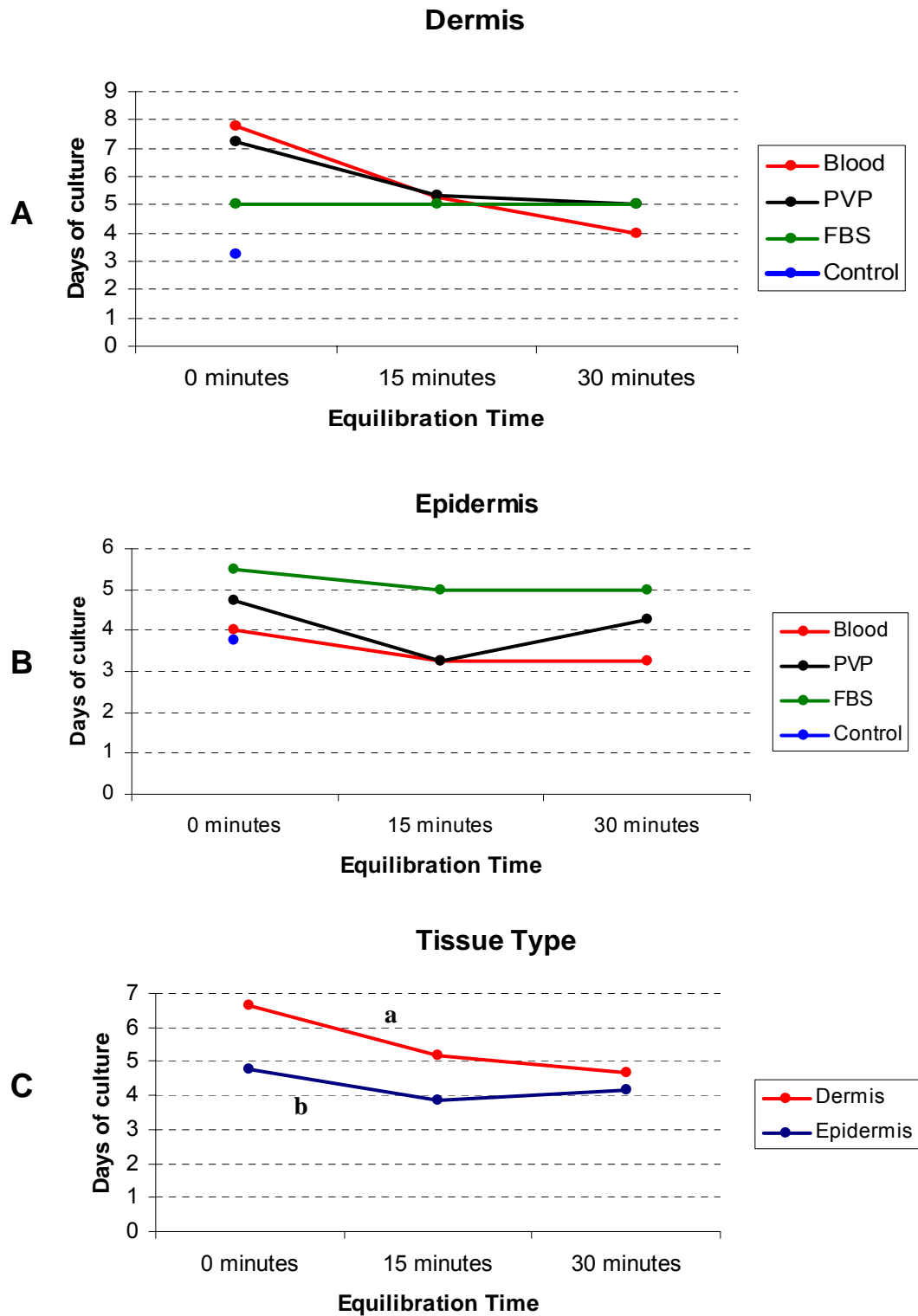


Figure 3.2. Days on which cells were observed in primary cultures compared within dermis (A) and epidermis (B) and across tissue types (C) of tissues frozen in different freezing media using three different equilibration times.

<sup>ab</sup> Values with different superscripts are statistically different (MANOVA,  $P < 0.05$ ). The 0 minutes of equilibration time group in the dermis tissue was different from that of the fresh control biopsies.

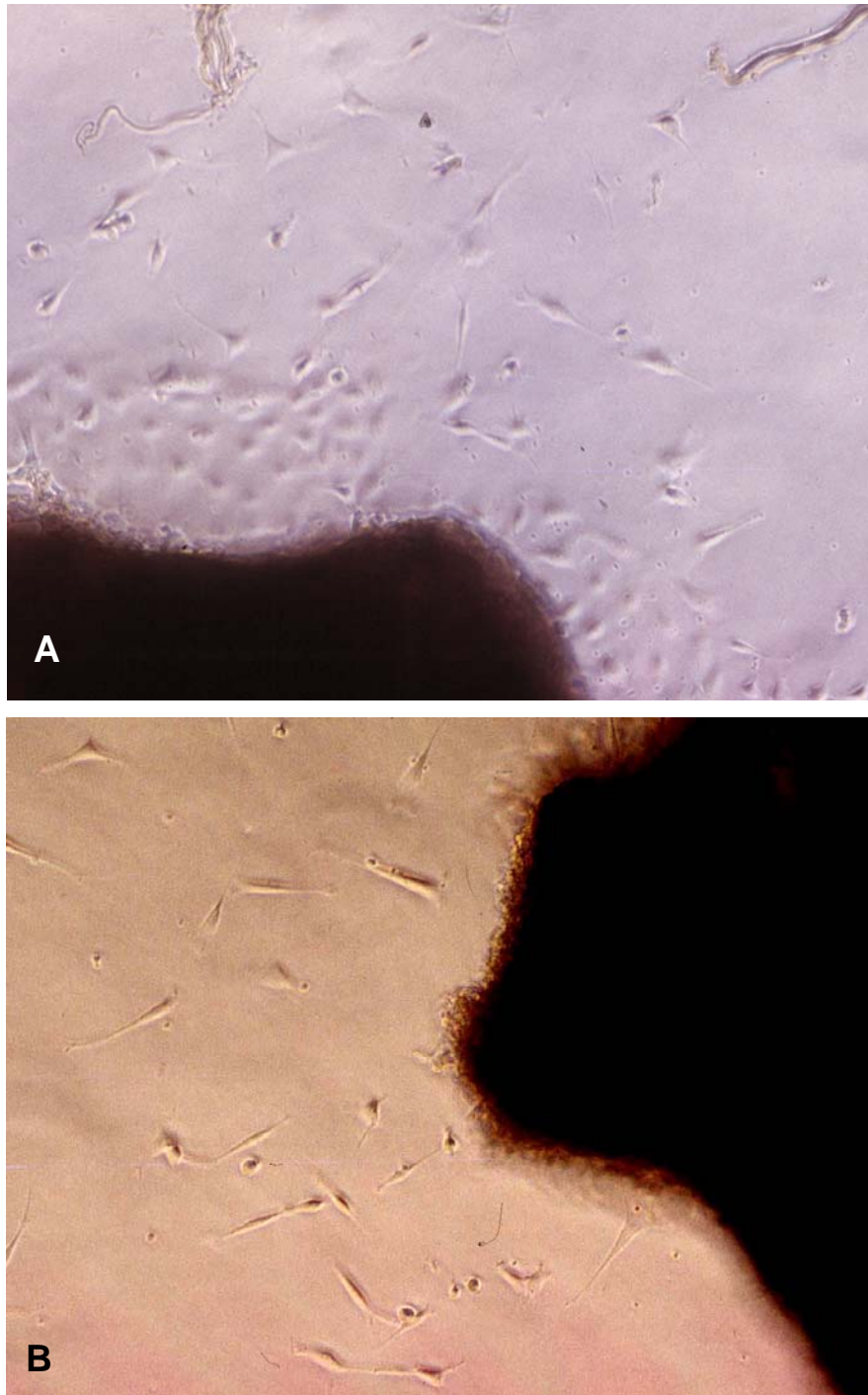


Figure 3.3. Epithelial and fibroblasts cells growing from the epidermal layer (A) and fibroblast cells growing from the dermal layer (B) of a frozen-thawed ram skin biopsy.

observed in culture), the 0-minute equilibration time group was significantly different from the fresh control group in the dermis ( $P<0.05$ ), whereas, the 15- and 30-minute equilibration groups were comparable with that of the control biopsies.

When the second variable was evaluated (day of primary culture confluence), significant differences were detected among medium groups ( $P<0.0001$ ) and tissue groups ( $P=0.0005$ ) but no significant differences were found among equilibration time groups ( $P=0.06$ ). Duncan's multiple range test indicated that blood and FBS media protected cells more effectively than PVP medium (Figures 3.4 A and 3.4 B) and less time was required for confluence to be reached in epidermal cultures than in dermal cultures (Figure 3.4 C).

For the third variable (day of passage 1 confluence), significant differences were found between medium ( $P<0.0001$ ) and tissue ( $P=0.0003$ ) type groups, but not between the equilibration time group ( $P=0.15$ ). Blood and FBS were not different, but it took more days for confluence to be reached in the PVP group (Figures 3.5 A and 3.5 B). The epidermal-derived cells reached confluence before those of the dermal-derived cells (Figure 3.5 C).

### **3.4 Discussion**

Cryopreservation of various tissue types in humans and animal has been in practice since the early 1900s. Applications range widely from grafting (Collier et al., 1987; Almodin et al., 2004) to cytochemical studies by electron microscopy (Bernhard and Viron, 1971) to simply reducing work load in tissue culture laboratories (Taylor et al., 1978; Gray et al., 1995). The numerous tissue types that are cryopreserved includes ovarian (Candy et al., 1997; Gook et al., 1999; Demirci et al., 2001; Almodin et al., 2004; Snow et al., 2004), testicular (Hovatta et al., 1996; Shinohara et al., 2002; Keros et al., 2005), brain (Collier et al., 1987, 1993; Fang and Zhang, 1992; Dong et al., 1993; Negishi et al., 2002) and heart (Sommer et al., 1999; Birtsas and Armitage, 2005), in addition to skin tissue (Taylor et al., 1978; Sommer et al., 1999; Silvestre et al., 2002, 2003, 2004), only to mention a few.

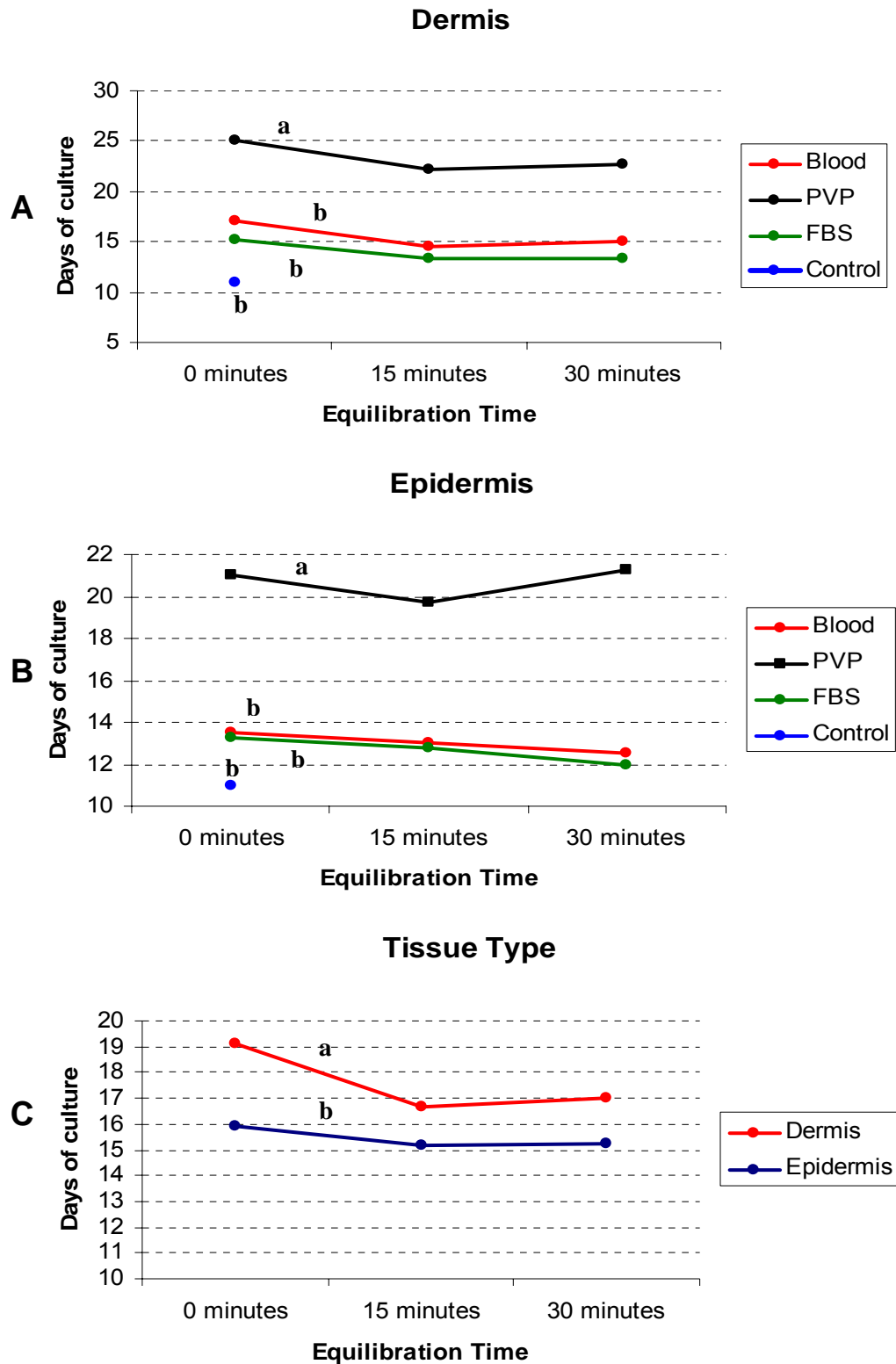


Figure 3.4. Days on which 75% confluence was reached in primary cultures compared within dermis (A) and epidermis (B) and across tissue types (C) of tissues frozen in different freezing media using three different equilibration times.  
<sup>ab</sup> Values with different superscripts are statistically different (MANOVA,  $P < 0.05$ ).

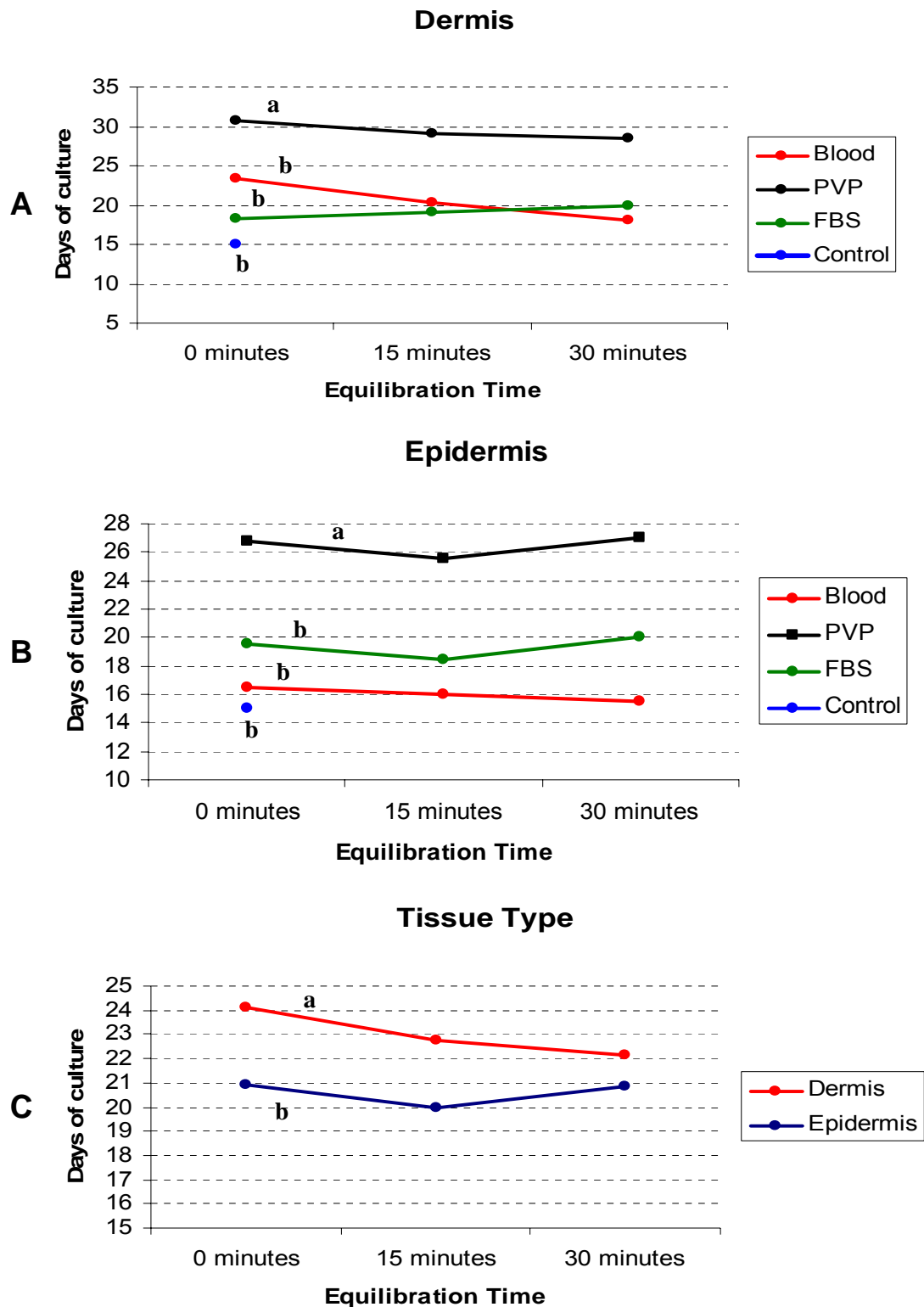


Figure 3.5. Days on which 90% confluence was reached in the first passage cultures compared within dermis (A) and epidermis (B) and across tissue types (C) of tissues frozen in different freezing media using three different equilibration times.  
<sup>ab</sup> Values with different superscripts are statistically different (MANOVA,  $P < 0.05$ ).



During the present study, skin tissue was frozen using several protocols. Cell lines were derived from all treatment groups, regardless of medium, equilibration time and tissue type. These results are similar to those for freezing rabbit and pig skin samples (Silvestre et al., 2002). Rendal Vázquez et al. (2005) using flow cytometry, reported that the viability of porcine heart valve cells was reduced from 90% in fresh valves to 56% in cryopreserved valves.

Although the fresh control biopsies in the present study needed the least amount of time for cells to grow, no significant differences between control and frozen biopsies were detected when medium or equilibration time groups were compared ( $P>0.05$ ). This may likely be attributed to the low number of samples used in the experiment.

The equilibration results were similar to findings by Snow et al. (2004) who reported that the equilibration time of mouse pup ovaries in DMSO prior to cryopreservation did not affect fertility of the ovarian tissue after grafting, as measured by the ability of ovaries to regain function and produce offspring. Ovaries were equilibrated 0, 30 and 120 minutes at 0°C or 120 minutes at 20°C.

In the present study, equilibration occurred at outside temperature (24 to 28°C) and time allowed for equilibration did not exceed 30 minutes. When medium types were pooled, however, the 0-minute equilibration samples required significantly more time to produce cell growth when compared with the fresh control tissue. DMSO is a membrane-permeable agent that protects cells from freezing injury on a colligative basis. Therefore, it is essential that the cryoprotectant is allowed to penetrate the tissue to reach and enter the cells to exert its protective action during the freezing process and prevent ice crystal formation. In the 0-minute equilibration group the cryoprotectant possibly could not penetrate the tissue thoroughly before submersion into liquid nitrogen and a portion of the cells remained unprotected, leaving a smaller population of viable cells to establish the cell line post-thaw. Based on these results, 15 minutes was enough time for equilibration to take place and no significant differences were observed between control, 15-minute and 30-minute groups.

Carsi et al. (2004) examined DMSO permeation through human articular cartilage as affected by temperature. They used cartilage samples much larger (10x30x2.2 to 3.4 mm) than our biopsies that had a 6 mm diameter and of various thicknesses (1 to 3 mm). Results showed that at 27°C the half-penetration time ( $t_{1/2}$ , the time required for 50% of the changes to occur and the recommended lowest limit for cryoprotection procedures) is around 21 minutes. It is therefore expected that our smaller tissue samples with a greater surface:volume ratio (1.67 vs. 1.18) should require less time for DMSO to permeate the fresh tissue.

When tissue types were compared, the epidermal layer required significantly less time to produce attached cells on the culture surface ( $P < 0.05$ ). This may simply be a factor of the thickness of the tissue type, with epidermis being thinner than the dermal tissue and therefore the epidermis was possibly more thoroughly equilibrated than the dermal tissue layer with the cryoprotectant by the time the tissue was frozen. As for the medium groups, cell lines were successfully derived after cryopreservation in all three medium types. The cryoprotective properties of DMSO was discovered almost 50 years ago (Lovelock and Bishop, 1959) and has been in used extensively for cryopreservation purposes ever since. This also holds true for many biological and nonbiological macromolecules like proteins and synthetic polymers, as discussed by some researchers in cryobiology in 1977 (Elliott and Whelan, 1977). Therefore, all medium types included 10% DMSO and a protein (serum) or a polymer (PVP).

Saline supplemented with 10% FBS and DMSO served as a conventional freezing medium control, since the most typical freezing medium usually contains serum or bovine serum albumin (BSA). Cell lines were obtained after thawing and processing from all biopsies frozen in DPBS with 10% FBS.

Blood, which is essentially a suspension of blood-bound cells in 100% serum, was successful in cryopreserving biopsy cells when 10% DMSO was included. Although not statistically different, it took the least amount of time for confluent cell monolayers to be derived from tissue frozen in blood when compared with DPBS supplemented with either FBS or PVP.

This could have valuable implications for field biologists who may not have other media available. During the present study, cryovials containing the appropriate volume DMSO were prepared and could potentially be carried on field expeditions without keeping them cool. One disadvantage of using the blood of the animal is that when tissue is collected post-mortem, the blood may already have clotted in the post-mortem animal. Thus, the technique would probably only be useful where the biopsy is collected from a live animal. Another aspect to consider is the possibility of blood-borne pathogens (Guertler, 2002) that may complicate transporting samples across borders. In such cases, nonbiological media have an advantage over the more traditional biological media.

It has been shown that PVP, a polymer that is membrane-impermeable, can act as a cryoprotectant by altering physical properties of extracellular solutions (Bricka and Bessis, 1955; Connor and Ashwood-Smith, 1973). Although more days were required for confluence to be reached, there was again no statistical difference and all frozen-thawed biopsies resulted in cell lines. Serum and blood contains fatty acids and lipid components which may help repair damaged membranes (Elliott and Whelan, 1977), whereas, this would not be true in the PVP group. There are, however, other properties that could make the synthetic polymer the cryoprotectant of choice for field biologists. PVP does not require refrigeration and the prepared medium could be transported during field research studies, which have major practical advantages over biological media. The possibility of preserving blood-borne pathogens with the tissue would also be reduced, especially when the biopsy is rinsed in an antibiotic solution before submersion into the PVP cryopreservation medium. For tissue that would be cultured in serum-free medium, the PVP medium may be of use to prevent the introduction of serum to the sample (Merten et al., 1995).

The focal purpose of this investigation was to develop a method for field biologists to cryopreserve biological samples in the field with minimum equipment and supplies. There is often no electric or gas-driven cooling or freezing units available, while portable LN<sub>2</sub> tanks are

more likely to be present. When used properly, these tanks can also hold LN<sub>2</sub> for extended periods of time. Furthermore, the need to preserve degradable biological medium during field expeditions could be a major hazard and if it is not kept cooled for a very short time, it has to be discarded and is an economical waste. When serum-free medium or the animal's own blood is used, this problem is eliminated since DMSO, glycerol and other cryoprotectant agents can be kept for extended periods without refrigeration. Cryopreservation vials containing PVP can be prepared ahead of time and transported to the field without the risk of degradation. In cases where blood from the tissue donor is used, tubes containing a known volume of a cryoprotective agent like DMSO or glycerol could be prepared and transported to the field, again with no need to keep it cool.

Therefore, this technique offers researches a choice of three different media that can be selected according to specific needs and circumstances, and may have valuable implications for endangered species conservation in the future.

## CHAPTER 4

### QUALITY AND FREEZING QUALITIES OF FIRST AND SECOND EJACULATES COLLECTED FROM ENDANGERED GULF COAST NATIVE RAMS\*

#### 4.1 Introduction

The Gulf Coast Native (GCN), also commonly referred to as the Louisiana Native, originated from a domestic population of sheep that originated in Europe. Following transplantation to the southern Gulf Coast Region of the United States in the 1500s, the breed became adapted to the warm humid environment climate of southern states (Mason, 1988). GCN sheep are characterized as having a degree of natural resistance to parasites, especially gastrointestinal nematode parasites, such as *Haemonchus contortus* (Miller et al., 1998; Li et al., 2001), as well as foot rot (Whittington et al., 1995), which often plague other sheep breeds in moist environments. As the sheep industry becomes revitalized in the southern United States, there has been increased interest in this endangered breed as part of crossbreeding programs to introduce these valuable characteristics to other wool and hair sheep breeds (Li et al., 2001). To ensure genetic diversity and long-term survival of this endangered breed, protection of the current genetic stock will be critical.

An important way of maintaining genetic diversity is by collecting and storing semen from many individuals for long periods (Frankham, 1996). In cattle, semen can be collected with an artificial vagina over multiple consecutive ejaculations without showing an apparent reduction in fertility (Seidel and Foote, 1969), but the collection of two ejaculates within a short period of time often affects seminal volume and sperm concentration. Similarly, in the domestic sheep, seminal volume and sperm motility have been shown to increase if there was at least a 3-day period between each collection (Ollero et al., 1996).

\* Reprinted from Animal Reproduction Science, Nel-Themaat, L., Harding, G.D., Chandler, J.E., Chenevert, J.F., Damiani, P., Fernandez, J.M., Humes, P.E., Pope, C.E., Godke, R.A., Qualities and freezing qualities of first and second ejaculates collected from endangered Gulf Coast Native rams, Electronic publication ahead of print., Copyright (2005), with permission from Elsevier.

To our knowledge, the effect of multiple electroejaculations in a single day with a short abstinence period between ejaculations has not been an area of investigation in the GCN sheep. Therefore, the aim of this study was to compare ejaculate volume, sperm concentration, number of sperm per ejaculate and progressive motility from first and second ejaculates collected 10 minutes apart on pre-cooled, cooled and frozen-thawed semen samples collected 3 days-a-week over a 3-week interval from GCN rams.

## **4.2 Materials and Methods**

### **▪ Experimental design**

A total of 74 ejaculates (38 first and 36 second ejaculates) were collected from five rams. Each collection day, two collection procedures were performed and the first and second ejaculates were compared. The variables that were measured were (1) ejaculate volume, (2) semen concentration, (3) number of sperm per ejaculate, (4) pre-cooled sperm motility, (5) cooled sperm motility and (6) post-thaw sperm motility.

### **▪ Animals**

GCN rams (n=5), ranging between 10 and 36 months of age and with body condition scores ranging between 2.75 and 3.50 on a scale of 1 to 5 (Russell, 1991), were obtained on loan from a nearby Gulf Coast Native sheep flock during the spring breeding season (late March and early April). The rams were all maintained on a Coastal bermudagrass pasture in a quarantine paddock at the LSU Agricultural Center Sheep Research Unit in Baton Rouge, Louisiana. The rams were allowed to acclimate after arrival for a period of 7 days before semen collection procedures were conducted for this study. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Agricultural Center (LSU).

#### ▪ **Chemical reagents**

All chemical reagents used in this study were obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

#### ▪ **Semen extender and cryoprotectant**

A standard Salamon's Tris-glucose-egg yolk extender was prepared as described by Evans and Maxwell (1987). Briefly, a stock solution of semen extender containing 36.34 mg/mL of Tris {hydroxymethyl} aminomethane, 5 mg/mL of glucose, 19.9 mg/mL of citric acid (Mallinckrodt, Hazelwood, MO, USA), 1,000 IU/mL of penicillin-G and 1 mg/mL of streptomycin sulfate was prepared and stored at 4°C. The prepared extender was subsequently used within 30 days of preparation.

Cryoprotectant semen extender (CSE) was prepared within 12 hours prior to semen collection by supplementing the stock solution with 15% egg yolk and 5% glycerol.

#### ▪ **Semen collection**

During the week prior to the initiation of the 3-week collection experiment, semen was collected via electroejaculation from each of the GCN rams and discarded as previously recommended (Evens and Maxwell, 1987). During the experiment, each ram was collected 3 days per week (Monday, Wednesday and Friday), with 2 to 3 days of rest between each semen collection procedure over a 3-week period. On each collection day, two ejaculates per ram were obtained by electroejaculation ~10 minutes apart. Each ejaculate was collected in a sterile 15-mL tube placed inside a 50-mL filled with water at 39°C to serve as a water jacket.

Although all rams were electroejaculated the same number of times in this experiment, some rams did not produce an ejaculate at every collection attempt, thus, the number of replicates were not equal for all five rams collected. Furthermore, when the volume of an ejaculate was very small, the semen sample was evaluated but not subjected to cryopreservation in this study.

#### ▪ Semen evaluation and cryopreservation

After each semen collection, the volume of each ejaculate (volume of ejaculate number 1 =  $V_1$  and volume of ejaculate number 2 =  $V_2$ ) was recorded and each ejaculate was placed into a 30°C water bath. At that time, a 10- $\mu$ L sample was diluted in 5 mL of 2.9% (v/v) sodium citrate solution to determine sperm concentration (ejaculate concentration number 1 =  $C_1$  and ejaculate concentration number 2 =  $C_2$ ) using spectrophotometry (Milton Roy Spectronic 20 spectrophotometer). The concentration was based on a standard curve established earlier by determining the concentration of different Gulf Coast Native ram semen samples ( $n=19$ ) with a standard bright-line phase hemocytometer (American Optical, Buffalo, NY, USA).

The ejaculate was then extended with fresh CSE 1:1 (v/v) at 30°C and placed with the water jacket into a styrofoam container filled with water at 30°C to slow down the cooling rate and prevent cold shock during transport to the cryopreservation laboratory (10 minutes from the semen collection area). By the time the semen reached the laboratory the water in the container as well as the semen had cooled down to ambient temperature.

To determine sperm pre-cooled progressive motility (pre-cooled semen progressive motility for ejaculate number 1 =  $PM_1$  and pre-cooled semen progressive motility for ejaculate number 2 =  $PM_2$ ), 50  $\mu$ L of an extended semen sample (22°C) was diluted with 1 mL of glycerol-free CSE in a 1.5-mL micro-centrifuge tube. A 15- $\mu$ L aliquot was then placed on a warmed (37°C) microscope slide and covered with a cover slip before examination under a phase-contrast microscope at 400X magnification. After observing four different fields, the percentage of progressively motile sperm was recorded for each sample. Throughout the experiment, the same technician evaluated all the samples without knowing which male produced each of the ejaculates.



Samples were evaluated for percent abnormal sperm and stained with eosin-fast green to evaluate if there were any detectable differences in the acrosome status between sperm from the first and second ejaculates across ram collections.

Semen samples were then slowly cooled to 5°C before warming and evaluating cooled progressive sperm motility (cooled motility for ejaculate 1 = CM1 and cooled motility for ejaculate 2 = CM2). After 1 to 2 hours of equilibration at 5°C, each sample was further diluted gradually with CSE (3 to 4 additions) to a final concentration of  $200 \times 10^6$  motile sperm/mL. The final CSE glycerol concentration ranged from 4 to 5%. The extended samples were then allowed to further equilibrate for a total CSE exposure of 4 hours. Semen samples were then loaded into 0.5 mL plastic straws (IMV, Maple Grove, MN USA) and after ultrasonical sealing, each straw was placed into the chamber of a freezing unit at -90°C. Straws containing extended semen samples were cooled to -150°C over a 15-minute interval using liquid nitrogen (LN<sub>2</sub>) vapor. The straws were then plunged into LN<sub>2</sub> for storage.

#### ▪ **Evaluation of post-thaw semen quality**

After 1 to 2 weeks of cold storage, semen samples were thawed by submerging each straw into a 39°C water bath for 15 seconds. Then, the sample was emptied into a 1.5-mL micro-centrifuge tube and 1 mL of phosphate-buffered saline (PBS; Gibco, Carlsbad, CA, USA) was added before determining post-thaw progressive motility (post-thaw motility for ejaculate number 1 = PTM1 and post-thaw motility for ejaculate number 2 = PTM2). Loss in progressive motility was subsequently determined by subtracting the cooled motility from pre-cooled motility, post-thaw motility from cooled motility and post-thaw motility from fresh motility to get the percentage of sperm cells that lost progressive motility during cooling (cooled sperm cell loss for ejaculate number 1 = CL1 and cooled sperm cell loss for ejaculate number 2 = CL2), during the freezing process (post-freezing loss for ejaculate number 1 = FL1 and post-freezing loss for

ejaculate number 2 = FL2) as well as overall loss in progressive motility (overall loss for ejaculate number 1 = OL1 and overall loss for ejaculate number 2 = OL2).

#### ▪ **Statistical analysis**

Within animal variation for the different variables was tested using the Statistical Analysis System package (SAS Institute, Inc., Cary, NC, USA). A two-way Analysis of Variance (ANOVA) was used to test for individual animal effects and to evaluate for variation between replicates using a General Linear Model (GLM). Paired t-test software procedures (Graphpad InStat, Graphpad Software, Inc., San Diego, CA, USA) were used to test for mean differences in semen/sperm parameters between first and second ejaculates across all rams and ejaculates. Comparisons for individual semen/sperm parameters were also evaluated for the first and second ejaculates across time (replications across time) by multiple linear regression using Graphpad InStat. Data are presented as mean $\pm$ SE, with a P-value of  $\leq 0.05$  considered to be a significant difference in this study.

### **4.3 Results**

In this study, 82 ejaculate attempts from five mature rams over 3-week interval resulted in 38 successful ejaculation procedures from rams at the first attempt and 36 successful ejaculation procedures for the second attempt 10 minutes later, resulting in a total of 74 (90.2%) successful ejaculation attempts for semen evaluation. Six of the missing collections (from one collection day) were the result of a malfunction of the electroejaculator (low battery power). In addition, during two of the ejaculation attempts, low-volume semen samples were obtained from two different rams and thus, were not subjected to the cryopreservation procedure.

The overall mean volume of semen collected per ram in the first ejaculate (1.62 mL) was significantly greater than the volume of semen collected per ram in the second ejaculate (1.06 mL) ( $P \leq 0.01$ ) (Table 4.1). Correspondingly, overall sperm concentration and total sperm per ejaculate were greater ( $P \leq 0.01$ ) in the first ejaculate ( $3.2 \times 10^9/\text{mL}$  and  $5.4 \times 10^9$ ) than in the

Table 4.1. Semen/sperm parameters, presented as pooled means ( $\pm$ SE), of the first and second ejaculates collected 10 minutes apart from five Gulf Coast Native rams.

Parameter	Ejaculate No. 1	Ejaculate No. 2
Volume, mL	1.62 $\pm$ 0.11 <sup>a</sup> (38)	1.06 $\pm$ 0.12 <sup>b</sup> (36)
Concentration, no. x 10 <sup>9</sup> /mL	3.2 $\pm$ 0.29 <sup>a</sup> (38)	1.5 $\pm$ 0.19 <sup>b</sup> (36)
Sperm/ejaculate, no. x 10 <sup>9</sup>	5.4 $\pm$ 0.70 <sup>a</sup> (38)	1.8 $\pm$ 0.50 <sup>b</sup> (36)
Pre-cooled sperm motility, %	71.5 $\pm$ 1.92 <sup>a</sup> (38)	75.0 $\pm$ 1.41 <sup>b</sup> (36)
Cooled sperm motility, %	64.8 $\pm$ 2.46 <sup>a</sup> (38)	72.4 $\pm$ 1.91 <sup>b</sup> (36)
Post-thaw motility, %	34.1 $\pm$ 3.12 <sup>a</sup> (37)	44.1 $\pm$ 3.07 <sup>b</sup> (28)

The number in parentheses represents the total number of samples for all rams.

<sup>ab</sup> Mean values in rows with different superscripts are significantly different ( $P \leq 0.01$ ).

second ejaculate ( $1.5 \times 10^9/\text{ml}$  and  $1.8 \times 10^9$ , respectively). In contrast, the mean percent progressive motility from the pre-cooled (22°C), cooled (5°C) and frozen-thawed spermatozoa were significantly lower ( $P \leq 0.01$ ) in the first ejaculate (71.5, 64.8 and 34.1%, respectively) than those of the second ejaculate (75.0, 72.4 and 44.1%, respectively) (Table 4.1). Furthermore, there was a tendency for more ejaculate to ejaculate variability in sperm concentration, sperm per ejaculate and in the progressive motility of pre-cooled, cooled and post-thawed sperm in the first ejaculate than in the second ejaculate collected 10 minutes later over a 3-week collection period.

When mean values for days of collection were compared across time (all replications) there were not significant differences for semen volume per day of collection for the first and second ejaculates, or sperm concentration for the first ejaculate per day of collection. There was, however, a significant decline in sperm concentration per day of collection for the second ejaculate ( $P \leq 0.05$ , Table 4.2). When mean values for days of collection were compared across time, there was not a significant difference in the number of sperm per ejaculate for the first and second ejaculates across replicates over time.

When mean values for days of collection were compared across time (all replications) there was a significant increase in pre-cooled progressive motility per day of collection for both the first and the second ejaculates over time ( $P \leq 0.05$ ) (Table 4.2). There was no significant difference for cooled progressive motility per day of collection for the first ejaculate, however there was a significant increase ( $P \leq 0.05$ ) in cooled progressive motility per day of collection over time for the second ejaculate. Furthermore, when mean values for days of collection were compared across time, there was not a significant difference in the post-thaw progressive motility per day of collection for either the first ejaculates or the second ejaculates across time. There was a significant reduction in the overall progressive motility from the pre-cooled (22°C) to cooled (5°C) and the cooled to the post-thaw motility for the first ejaculate ( $P \leq 0.01$ ),

Table 4.2. Gulf Coast Native ram semen parameters (mean±SE) for the first and second ejaculates over a time span of seven collections.

Parameter	Ejac*	Collection days (Replication, no.)						
	no.	1	2	3	4	5	6	7
Volume	1	1.80±0.3	1.50±0.1	1.58±0.2	1.94±0.3	1.00±0.1	1.60±0.2	1.96±0.5
(mL)	2	0.90±0.2	0.80±0.1	0.97±0.1	0.80±0.1	0.92±0.2	1.63±0.8	1.34±0.3
Concentration	1	3.64±0.5	3.99±0.8	3.63±0.9	4.08±0.7	1.74±0.5	2.27±0.7	3.18±0.8
(no. x 10 <sup>9</sup> )	2 <sup>a</sup>	2.13±0.4	2.22±0.3	1.15±0.5	1.97±0.6	1.10±0.3	1.16±0.8	1.08±0.3
Sperm/Ejac	1	6.56±1.4	6.15±1.5	6.18±1.9	7.72±1.4	1.77±0.5	4.03±1.6	6.58±0.9
(no. x 10 <sup>9</sup> )	2	1.25±0.7	1.74±0.1	1.25±0.6	1.55±0.7	1.24±0.5	3.90±3.6	1.07±0.7
Pre-cooled	1 <sup>b</sup>	61±2.4	71±3.8	64±2.4	75±3.2	81±2.4	72±8.5	74±4.0
motility (%)	2 <sup>b</sup>	70±1.6	72±1.7	72±2.5	73±3.2	83±2.0	79±6.6	76±3.3
Cooled motility	1	49±4.3	70±4.6	59±1.9	68±5.6	72±6.0	63±9.3	71±5.3
(%)	2 <sup>c</sup>	68±2.0	70±2.9	69±2.4	71±4.7	73±5.1	71±8.7	83±4.9
Post-thaw	1	20±7.4	46±3.8	28±7.2	40±10.5	38±8.2	26±10.7	38±5.6
motility (%)	2	50±1.8	53±6.0	48±9.3	34±14.2	45±7.6	40±10.0	40±2.0

\* Ejac no.=first or second ejaculates.

<sup>a</sup> There was a significant decrease in sperm concentration across collection days (replications) ( $P \leq 0.05$ ).

<sup>b</sup> There was a significant increase in pre-cooled progressive motility for both the first and second ejaculates across collection days (replications) ( $P \leq 0.05$ ).

<sup>c</sup> There was a significant increase in pre-cooled progressive motility for the second ejaculate across collection days (replications) ( $P \leq 0.05$ ).

however, a significant reduction was only detected from the pre-cooled to the post-thaw progressive motility for the second ejaculate ( $P \leq 0.01$ ) (Figure 4.1). The loss in progressive motility from the pre-cooled semen to the cooled semen was greater ( $P \leq 0.05$ ) in the first ejaculate at 6.7% than that of the second ejaculate at 2.6% (Figure 4.2), however, the loss of progressive motility from cooled semen to that of post-thawed semen was not significantly different ( $P > 0.05$ ) when the first ejaculate was compared with that of the second ejaculate (29.1% vs. 26.1%, respectively).

Sperm morphology (% abnormal sperm) was not found to be different between the first ejaculate and the second ejaculate and there was not a detectable difference in sperm morphology for ejaculates across days of collection (replications) in this study.

#### **4.4 Discussion**

The results of this experiment demonstrate that it is possible to successfully collect a second ejaculate from GCN rams within a short interval (10 minutes) after collection of the first ejaculate for cryopreservation purposes. To our knowledge, this is the first study to compare successive ejaculates obtained by electroejaculation in GCN rams.

In the domestic ram, collection of multiple ejaculates significantly reduced the sperm concentration (Salamon, 1962, 1964; Amir and Ortavant, 1968; Jennings and McWeeney, 1976) and the semen volume and concentration were dependent on abstinence period (Chang, 1945; Ollero et al., 1996). Similarly, in the present study, we found that the overall semen volume, sperm concentration and the total number of spermatozoa per ejaculate were decreased in the second ejaculate when collection was executed 10 minutes after collection of the first ejaculate. In cattle, semen volume and sperm concentration in the second ejaculate were also found to be reduced when the ejaculates were collected within an interval of 40 minutes or less from the first ejaculate (Seidel and Foote, 1969), but when the interval between ejaculates was increased to 1 hour, it was found that the semen volume of the second ejaculate from the same bulls was not

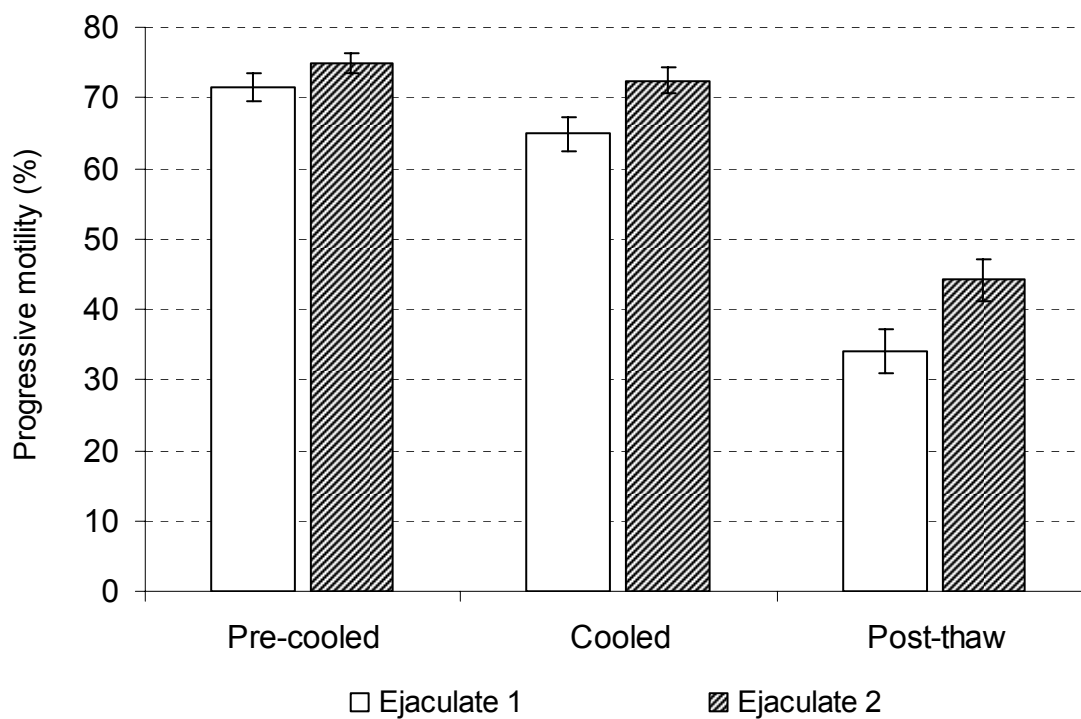


Figure 4.1. Percent progressive motility at different stages of the cryopreservation process for first and second ejaculates collected 10 minutes apart from five Gulf Coast Native rams over a 3-week period.

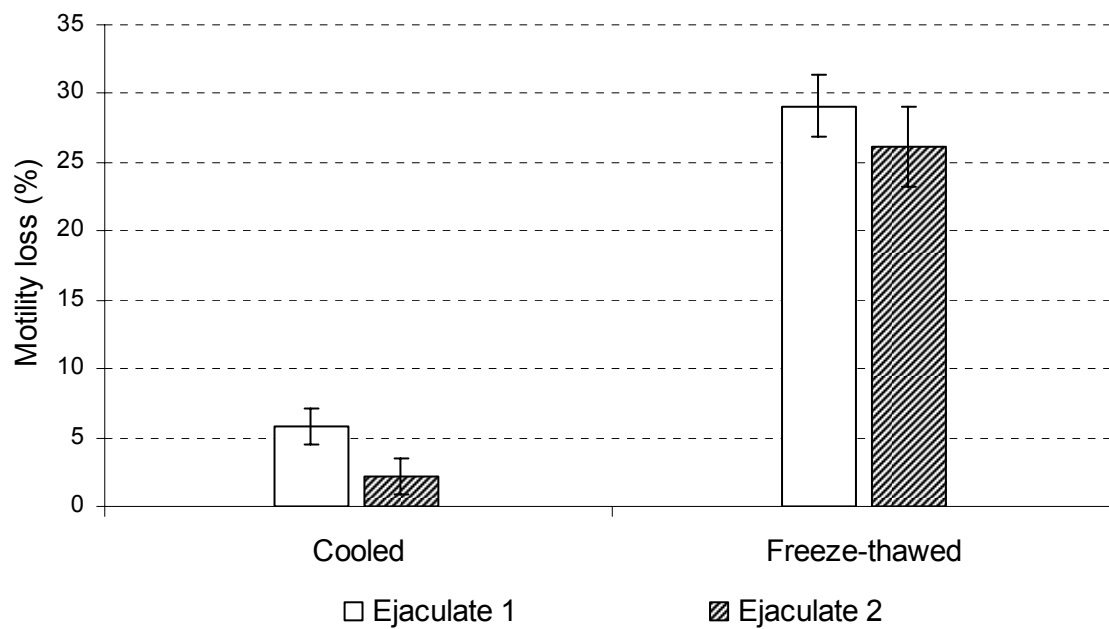


Figure 4.2. Percent loss in progressive motility at different stages of the cryopreservation process for first and second ejaculates collected 10 minutes apart from five Gulf Coast Native rams over a 3-week period.



reduced, even though sperm concentration in the second ejaculate was found to be lower (Pickett and Komarek, 1967).

Not only can the total number of spermatozoa in the ejaculate be affected by the abstinence period and the number of ejaculates in rams as previously reported (Ollero et al., 1996), but can also be affected by the interval between successive ejaculates. Further studies, however, are needed on the interval between successive ejaculates in both the GCN and other domesticated rams to determine the collection interval required to maintain the semen volume and sperm concentration when collecting spermatozoa by electroejaculation.

The results obtained on progressive motility of first and second ejaculates collected by an artificial vagina in sheep have been contradictory. Studies have reported no differences in motility within two ejaculates when collected with an interval of 20 minutes (Salamon, 1964) or 8 hours (Salamon, 1962), while others have reported that progressive motility in the second ejaculate was greater than that in the first ejaculate, when the second ejaculate was collected 24 hours later (Ollero et al., 1996).

Our study also found greater progressive motility in the second ejaculate for pre-cooled (22°C), cooled at 5°C and cryopreserved semen after thawing when compared with the progressive motility in the first ejaculate. Although the second ejaculate was collected 10 minutes after the first ejaculate in the present study, it is not clear why we found significantly higher proportions of progressively motile sperm, when other reports indicated no differences between ejaculates collected at intervals from 20 minutes up to 8 hours (Salamon, 1962, 1964). It is possible that the Gulf Coast Native sheep breed or possibly differences in the semen collection techniques may have affected post-collection sperm motility in the present study. The time that spermatozoa remain in the cauda epididymides and/or ampulla portion of the vas deferens before electroejaculation 2 or 3 days later may have contributed to the difference in progressive motility detected between the first and second ejaculates in the present study. Even if the reproductive tracts of the rams were or were not 'flushed' before the start of the collection

interval, it is proposed that the first ejaculate on each collection day over the 3-week interval would still have residual post-epididymal sperm from the semen collection 2 to 3 days earlier, whereas, this would not be the case for the second ejaculate collected 10 minutes later over this interval.

It is known that spermatozoa undergo final maturation and acquire functionality and motility during epididymal transit from the caput to cauda region of the epididymis. This is accompanied by biochemical changes, especially in the plasmalemma that has been found to undergo a reduction in phospholipids, cholesterol and fatty acid content (Quinn and White, 1967; Parks and Hammerstedt, 1985; Bedford and Hoskins, 1990). Prior to ejaculation, the mature sperm cells are generally stored in a quiescent state in the cauda epididymides as the main reservoir of spermatozoa available for ejaculation. As more maturing sperm enter the cauda portion, spermatozoa that are not ejaculated move passively into the vas deferens where they may start undergoing degradation without the protective influences from the caudal epithelium and testicular androgen (Bedford and Hoskins, 1990). A portion of cells from the previous ejaculate may also remain in the ampulla where more changes can take place until the spermatozoa are evacuated during the next ejaculation (Almquist and Amann, 1961). Therefore, a large fraction of the first ejaculates in our study may have consisted of “older” spermatozoa, as has been previously suggested by Bedford and Hoskins (1990).

The lipid content and dry weight of seminal plasma and spermatozoa have been previously compared from first and second ejaculates of mature bulls (Pickett et al., 1967). They reported that the seminal plasma from first ejaculates contained a higher percentage of lipids than that of the second ejaculate, however, the percentage of lipids in the spermatozoa was higher in the second ejaculate when compared with that of the first ejaculate. Furthermore, the reduction in lipid content of spermatozoa during epididymal transit and storage have also been reported to increase subsequent cold-shock sensitivity (Watson and Morris, 1987).

It is interesting to note that Quinn and White (1967) reported that ram spermatozoa collected from testes and caput epididymides were affected little by cold shock, whereas, spermatozoa collected from the cauda and ampulla had cold shock susceptibility that was similar to ejaculated ram spermatozoa. Thus, it seems possible that the second semen ejaculates from rams in our study contained a higher percentage of “younger aged” post-epididymal sperm that exhibited higher pre-cooled progressive motility and higher resistance to decreased temperatures, which may have resulted in a higher overall sperm post-thaw survival rate for sperm from that ejaculate. It is proposed that sperm from the second ejaculate would have less total post-epididymal maturation time *in situ* and this could affect post-thaw motility.

In the present study, the more prominent difference in progressive motility loss between the two ejaculates occurred during the cooling process (ambient temperature to 5°C) rather than during the freezing-thawing process. Therefore, it appears that most of the sensitive spermatozoa in the first ejaculate lose motility during the cooling process, rather than the freezing-thawing process. Furthermore, Holt et al. (1992) have reported that loss of membrane integrity during the re-warming process after cooling occurred at a lower temperature than when ram spermatozoa had been frozen. They postulated that ice crystal formation around the spermatozoa causes a considerably different type of cell damage than does that of cold shock produced by cooling. Thus, in our study, an alteration in sperm membrane structure between the spermatozoa of the first and second ejaculates may have resulted in different responses to cooling rather than the actual freezing of the spermatozoa, hence the lack of a difference in motility loss after freezing and thawing between the two ejaculates.

Tamuli and Watson (1994) reported that spermatozoa acquired an increasing resistance to cold shock progressively over a 24-hour period after ejaculation. They noted that acrosome damage of boar sperm began to occur after 16 hours. Should this apply for ram spermatozoa, the difference in response to cooling and freezing between the two ejaculates could also be due to the following events: First, by the time of freezing most of the “older aged”,

compromised spermatozoa were already nonmotile, which resulted in the percentage of “younger aged”, more resistant spermatozoa becoming more equal between the two ejaculates. Secondly, a 4-hour incubation interval could be enough for a larger proportion of the more susceptible, older aged spermatozoa in the first ejaculate to acquire enough cold resistance to withstand freezing. Our findings suggest that additional research is needed to evaluate biochemical composition and membrane alterations of ram spermatozoa during both the cooling and freezing processes for successive ejaculates collected during short-term intervals.

In conclusion, our results demonstrated that it is possible to successfully collect a second ejaculate from GCN rams within a short interval (10 minutes) after collection of the first ejaculate three times a week. This collection schedule could be continued for a 3-week period without any apparent decline in semen volume, total sperm per ejaculate or sperm quality. The only difference between two successive ejaculates over time (days of collection) was a decrease in sperm concentration for the second ejaculate in our study, which corresponds to the results for decreased sperm concentration and the response of spermatozoa to cooling and freezing reported for multiple ejaculates obtained from rams by an artificial vagina method (Salamon, 1962, 1964; Jennings et al., 1976; Ollero et al., 1996). Further studies on the interval between successive ejaculates and the length of the collection schedule should be conducted to determine the optimal collection schedule needed to maintain maximum semen volume, sperm concentration and total sperm output during a single collection interval to keep ram maintenance and handling time to a minimum.

The improvement in cooling and freezing resistance of the second ejaculate collected within 10 minutes of the first ejaculate may be of value, especially where high quality frozen-thawed spermatozoa are required for *in vitro* production of embryos. The fertilizing capacity of spermatozoa from both ejaculates after cryopreservation should be examined to validate the rationale for long-term storage of spermatozoa from a second ejaculate collected shortly after the first ejaculate. Finally, cryopreservation of semen from GCN rams for use in assisted

reproduction techniques, including artificial insemination (vaginal, cervical and intrauterine), *in vitro* fertilization and intracytoplasmic sperm injection could contribute towards ensuring the survival of this unique endangered sheep breed for future generations.

## CHAPTER 5

### ISOLATION, CULTURE AND CHARACTERIZATION OF SOMATIC CELLS DERIVED FROM SEMEN AND MILK OF NONDOMESTIC SPECIES

#### 5.1 Introduction

In the present study, attempts were made to isolate, culture and characterize somatic cells derived from (1) fresh, cooled and frozen-thawed semen of the Gulf Coast Native (GCN) sheep (*Ovis aries*) and fresh semen of an Eland antelope (*Taurotragus oryx*) and (2) fresh sheep milk. Also, an effort was made to determine the ability of Eland antelope somatic cells isolated from semen to dedifferentiate in eland cytoplasts after somatic cell nuclear transfer (SCNT).

Several mammalian species are facing the threat of extinction as a result of vanishing ecosystems, proliferation of human populations and an increase in human-mediated interferences (Blackburn, 2004). Declining population numbers of many threatened species have brought into prominence the importance of maintaining genetic diversity of remaining small populations with limited gene pools. This is extremely important in managing captive populations, in which new influx of genetic material is generally limited (Ryder et al., 1997). As a result, genome resource banking (GRB) has assumed an increasingly important role in the field of conservation biology. The ability to cryopreserve and store gametes (spermatozoa and oocytes) and embryos from genetically valuable animals are key components of biological resource banks (BRBs). However, gametes and embryos are finite in their use and have to be replaced constantly if used on a regular basis, which is not possible if donor animals are inaccessible or dead. Therefore, the cryopreservation of viable somatic cell tissues is quickly assuming an increasingly important role in BRBs (Ryder, 2002). Although these tissue samples are also finite, they can be propagated by using specific culture conditions before being cryopreserved and stored for many years (Green, 1967).

The birth of the first mammal following transfer of embryos derived by SCNT using an adult donor cell (Wilmut et al., 1997), demonstrated that the nucleus of an adult somatic cell maintains its totipotency. Since then, the totipotency of somatic cells from nondomestic species has also been demonstrated by producing viable cloned animals in, for example, the gaur (Lanza et al., 2000), mouflon (Loi et al., 2001), deer (<http://www.cnn.com/2003/TECH/science/12/22/cloned.deer.ap/index.html>), banteng (Janssen et al., 2004), African wildcat (Gómez et al., 2004) and Water buffalo (<http://www.chinaembassy.org>). These results indicated that SCNT is a potentially viable technique for the conservation of endangered species.

Fibroblast cells derived mostly from skin samples are widely used as somatic donor cells for SCNT. In addition, alternative sources of somatic cells include those derived from hair follicles (Arase et al., 1990; Kurata et al., 1994; Moll, 1996), colostrum (Kishi et al., 2000) or blood (Elicha Gussin and Elias, 2002). Freshly lactated milk and ejaculated semen contain large numbers of somatic cells (Paape and Tucker, 1966; Evenson and Melamed, 1983). Likewise, the totipotency after SCNT of mammary gland epithelial cells isolated from milk colostrum has been demonstrated by the birth of live cloned calves (Kishi et al., 2000). Somatic cells isolated from endangered species from milk and ejaculated semen should be considered as potential donor cell sources and included in BRBs.

## **5.2 Materials and Methods**

### **▪ Experimental design**

For ram semen, the cryopreservation treatments were: (1) fresh, (2) cooled, (3) -1°C/minute cooling rate and (4) -10°C/minute cooling rate. Variables measured were the number of attempts in which cells (a) attached, (b) proliferated, (c) proliferated until passage was performed and (4) proliferation after passage. A total of seven replicates (collections) were performed.

For milk, two breed types (GCN and GCN X Suffolk crossbred) were compared and the number of attempts in which cells (a) attached and (b) proliferated were measured. Cell lines obtained were characterized by immunohistochemical detection of vimentin and cytokeratin.

#### ▪ **Animals**

GCN rams and ewes, as well as GCN X Suffolk crossbred ewes, all between two and five years of age, were housed at the Louisiana State University (LSU) Agricultural Center Sheep Research Unit, while the eland bull was housed at the Freeport-McMoRan Audubon Species Survival Center (FMA-SSC). All sheep and eland procedures were approved by the Institutional Animal Care and Use Committee of LSU and FMA-SSC.

#### ▪ **Chemicals**

All chemicals, including antibodies for immunocytochemistry, were obtained from Sigma Chemical Company (St Louis, MO, USA), unless otherwise stated.

#### ▪ **Semen collection**

Semen was collected from two GCN rams and one 6-year old Common eland antelope bull with a body weight of ~530 kg (Figure 5.1). Ram semen was collected from two 4-year old (body conditions scores of 3 and 4) rams by electroejaculation (Evans and Maxwell, 1987), while eland semen was collected by a combination of rectal massage and electroejaculation (Wirtu et al., 2002b). Each ram semen sample was divided into four treatments before somatic cell isolation: (1) fresh, (2) cooled, (3) slow-freezing and (4) fast-freezing, while eland somatic cells were isolated from only fresh semen.

Treatment 1: For fresh samples, an aliquot (0.5 mL) of fresh ram (n=7) or eland (n=2) semen was diluted in the presence of seminal plasma with minimum essential medium alpha medium ( $\alpha$ -MEM, Gibco, Grand Island, NY, USA; see Appendix B2) containing 15% newborn calf serum (NCS, Gibco) and supplemented with 100 IU/mL of penicillin G and 0.1 mg/mL of streptomycin (10  $\mu$ L/mL; Cellgro, Herndon, VA, USA). Diluted semen was incubated at 39°C for





Figure 5.1. Two Gulf Coast Native (*Ovis aries*) rams, polled (A) and horned (B), and the eland (*Taurotragus oryx*) bull (C) that were used for semen collections during the study.

no more than 5 hours before somatic cell isolation. Treatment 2: After an aliquot of fresh semen sample was taken, each ejaculate was allowed to cool to 30°C and then extended in Salamon's standard Tris-glucose extender supplemented with 15% (v/v) egg yolk and 5% (v/v) glycerol (Evans and Maxwell, 1987). Additional extender was added to obtain a final concentration of  $200 \times 10^6$  spermatozoa/mL and a glycerol concentration of 4 to 5%. Then, extended semen was slowly cooled to 4°C for 2 hours. After cooling, approximately one third of the extended cooled semen was used for somatic cell isolation and the remaining two thirds of the sample were used for cryopreservation.

Treatment 3: For slow-freezing, cooled semen (one fourth of ejaculate) was loaded into 1.0 mL cryovials (1 mL/cryovial), and cooled at 1°C/minute to -80°C/minute (Mr. Frosty; Nalgene, Rochester, NY, USA) before storage in liquid nitrogen (LN<sub>2</sub>).

Treatment 4: For fast-freezing, extended semen (one fourth of ejaculate) was loaded into 0.5 mL straws (IMV, Maple Grove, MN, USA) (n=2 to 5 straws depending on ejaculate volume) and after sealing the tip, each straw was placed into the chamber of a controlled rate freezing unit (Model CL-863, Cryologic, Victoria, Australia) at 4°C, cooled at -10°C/minute to -80°C and plunged into LN<sub>2</sub> for storage.

#### ▪ **Milk collection**

Sheep milk samples (n=26) were collected by hand from pure GCN (n=5), and crossbred ewes (n=10). Two collections were performed from each animal, but contamination occurred in four samples from GCN ewes and these were therefore omitted from the experiment. After collection, milk was cooled to 4°C and stored for 12 hours.

#### ▪ **Somatic cell isolation, culture and cryopreservation**

Fresh (0.5 mL), cooled (third of extended ejaculate) and frozen-thawed semen (2 to 5 cryovials or straws) and cooled milk (15 to 50 mL) were washed in 10 mL of Ca<sup>2+</sup> and Mg<sup>2+</sup>-free Dulbecco's phosphate-buffered saline (DPBS, Gibco) and centrifuged (500 x g) for 10 minutes.

Pelleted samples of each semen treatment were resuspended in 2 mL of  $\alpha$ -MEM supplemented with 15% NCS, 1,500 IU/mL of penicillin, 5 mg/mL of streptomycin, 250  $\mu$ g/mL of gentamicin and 500 IU/mL of polymixin B, placed in a 60-mm tissue culture dish (Falcon 1007, Becton Dickinson & Company, Franklin Lakes, NJ, USA), covered with a layer of Type 1 collagen (from calf skin) and cultured at 38.5°C in 5% CO<sub>2</sub> in air. Pelleted milk was resuspended in 2 mL of Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 15% Fetal Bovine Serum (FBS), 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin, placed in 60 mm tissue culture dishes and cultured at 38.5°C in 10% CO<sub>2</sub> in air.

After 24 hours of incubation, dishes from each treatment were rinsed once and culture medium was replaced every 3 to 4 days. Dishes were periodically searched for the presence of attached cells using an inverted microscope (100X). When cell colonies covered  $\sim 0.5$  cm<sup>2</sup> of the dish surface (proliferated) they were disaggregated. Somatic cells obtained from semen samples were disaggregated by 15 minutes of incubation in PBS at 38.5°C, followed by 20 minutes of incubation in 200 IU/mL of collagenase. Somatic cells obtained from milk were dissociated by incubation in 0.25% trypsin until all the cells were dissociated from the surface of the culture dish.

Dissociated cells were centrifuged (500 x g) for 10 minutes, resuspended in  $\alpha$ -MEM (semen somatic cells) or DMEM (milk somatic cells) and cultured in 35-mm collagen-coated dishes or 75-cm<sup>2</sup> tissue culture flasks for sperm or milk-derived cells, respectively (passaged). When cells from each sample reached confluence, cells were passaged one or two times before being resuspended in tissue culture medium ( $\alpha$ -MEM or DMEM) with 15% NCS and 10% (v/v) dimethyl sulfoxide (DMSO) and cooled at 1°C/minute to -80°C (Mr. Frosty, Nalgene, VWR, Bridgeport, NJ, USA) before storage in liquid nitrogen.

#### ▪ Cell characterization and microsatellite analysis

Cell characterization was performed on cells that were obtained during preliminary studies from fresh and cooled GCN ram semen and from fresh eland semen during the current experiment. A fraction of cells from each established cell line was cultured on a microscope cover slip. Then, immunohistochemical detection of cell types was performed by fluorescent labeling of cytokeratin and vimentin for identifying epithelial and fibroblast cells, respectively (Katska et al., 2002). Briefly, cells were cultured on sterilized microscope coverslips or Biocoat™ collagen-coated culture slides (Becton Dickson & Company, Franklin Lakes, NJ, USA) and fixed in 3.7% paraformaldehyde (PF), permeabilized for 10 minutes in 1% Triton X-100 diluted in PBS (PBS-T), rinsed twice in 0.1% PBS-Tween 20 (PBS-T20) and stabilized with 0.1 M glycine solution. Primary monoclonal mouse antibodies were: (1) 1:100 anti- $\beta$ -tubulin (positive control), (2) 1:100 anti-vimentin (for labeling fibroblast cells) and (3) 1:200 anti-pan cytokeratin (for labeling epithelial cells).

Incubation of the somatic cells with the primary antibodies was performed on a shaking platform overnight at room temperature (RT). After incubation, somatic cells were rinsed three times for 10 minutes per rinse in PBS-T20.

A negative control was also included by labeling only with a secondary antibody that was added and incubated overnight on a shaking platform at RT. The  $\beta$ -tubulin and vimentin labeled slides were incubated in anti-mouse IgG-TRITC conjugate, while the negative control and cytokeratin labeled slides were incubated in anti-mouse IgG-FITC conjugate, both at 1:64 dilutions in PBS-T. The next day, slides were rinsed three times for 10 minutes per rinse in PBS-T20 before mounting in glycerol containing 0.5  $\mu$ g/mL of Hoechst 33342. Under epifluorescence microscopy, epithelial cells labeled for with cytokeratin fluoresced green while fibroblast cells labeled for vimentin fluoresced red.

To determine the genetic status of the established cell lines, DNA was extracted from a blood sample and epithelial cells from the semen sample of the eland sperm donor using a

Qiagen DNAeasy Kit (Qiagen, Valencia, CA, USA). DNA was also isolated from a blood sample of a second, unrelated eland. Fourteen microsatellite markers (BM1818, BM1824, BM2113, BRR, CYP21, ETH10, ETH225, INRA023, RM006, RM067, SPS115, TGLA122, TGLA126, TGLA227) that were derived from cattle were tested for efficacy in the eland ([www.isag.org.uk/ISAG/all/02\\_PVpanels\\_LPCGH.doc](http://www.isag.org.uk/ISAG/all/02_PVpanels_LPCGH.doc)) (Creighton et al., 1992; Kossarek et al., 1993a, 1993b; Bishop et al., 1994). These markers were fluorescently labeled for analysis with ABI instrumentation (Applied Biosystems, Foster City, CA, USA). Then ~10 ng of genomic eland DNA (2 µL of extracted DNA) was used per 10 µL PCR reaction. PCR parameters included an initial 3-minute denaturation at 94°C followed by 35 cycles of 1-minute denaturation at 94°C, annealing for 1 minute at 58°C and a 72°C extension for 1 minute, followed by a final extension at 72°C for 10 minutes. After the initial amplification, the products were denatured at 95°C for 3 minutes, PCR products were separated on an ABI Prism 377 DNA Analyzer after the addition of 2 µL of formamide loading dye with GeneScan 350 ROX size standard to ~0.1 µL of the PCR product. The allele sizes and genotypes were determined using the STRand software (Toonen and Hughes, 2001).

▪ **Oocyte maturation, nuclear transfer and embryo culture**

To obtain eland oocytes, two females were treated with altrenogest orally (0.044 mg/kg Regumate®, Intervet, Inc., Millsboro, DE, USA) for 7 days and prostaglandin F<sub>2</sub>α (25 mg, Lutalyse®, Pharmacia and Upjohn Company, Kalamazoo, MI, USA) was administered (intramuscular) on the last day of altrenogest treatment. Ovarian follicular stimulation was induced using porcine follicle stimulating hormone (equivalent to 400 mg NIH, Folltropin®, Bionche Animal Health Canada, Inc., Belleville, Ontario, Canada) dissolved in 30% polyvinyl pyrrolidone.

Oocytes were collected by transvaginal ultrasound-guided aspiration of mature ovarian follicles at 48 hours following prostaglandin treatment (Wirtu, 2004). Collected oocytes were subjected to *in vitro* maturation (IVM) in tissue culture medium 199 (TCM 199) supplemented

with 10% NCS, follicle stimulating hormone (FSH, Sioux Biochemical, Sioux Center, IA, USA), luteinizing hormone (LH, Sioux Biochemical), estradiol, pyruvate and epidermal growth factor (EGF) (Krisher and Bavister, 1999) at 39°C in 5% CO<sub>2</sub> in air. After 26 hours, cumulus cells of matured oocytes were removed with hyaluronidase (0.1%). Denuded oocytes were then placed in HEPES-buffered TCM 199 supplemented with 7.5 µg/mL of cytochalasin B, 1.0 µg/mL of Hoechst 33342 and 10% fetal bovine serum (FBS) for 20 to 30 minutes before enucleation.

The first polar body and the metaphase II plate were drawn into an enucleation pipette and successful enucleation was confirmed by epifluorescence microscopy. A single epithelial cell isolated from fresh eland semen was introduced into the perivitelline space of each enucleated oocyte by micro-injection. For fusion, couplets in Zimmermann fusion solution were placed in a fusion chamber (BTX, San Diego, CA, USA) with two electrodes, 1 mm apart. Cell fusion was induced with a single DC pulse of 1.75 kV/cm for 15 µseconds using a BTX Cell Manipulator 2001. Fused couplets were activated by culturing them in 5 µM ionomycin for 5 minutes and then in 1.9 mM 6-dimethyl-aminopurine (DMAP) for 3 to 4 hours. After activation, reconstructed embryos were cultured in embryo culture medium (ECM) consisting of α-MEM containing 1% MEM nonessential amino acids, 2% BME essential amino acids, 10% FCS and 0.25 mM pyruvate. Embryo development was evaluated on day 2 (cleavage) and day 7 (morula and/or blastocyst development). Embryo culture was conducted in a humidified atmosphere with 5% CO<sub>2</sub>, 10% O<sub>2</sub> and 85% N<sub>2</sub> at 39°C.

#### ▪ Embryo transfer

To evaluate the *in vivo* competence of cloned eland embryos, morulae and blastocysts were transferred into the uterus of each eland recipient (n=2) on day 7 or 8 of the estrous cycle. For transfer, epidural analgesia was administered to females after voluntarily entering the hydraulic restraining chute (Tamer™, Fauna Research, Inc., Red Hook, NY, USA) and the perivulvar area was cleaned before entry of the transfer catheter. The embryos were aspirated into a 0.25 mL plastic straw (AgTech, Manhattan, KS, USA) that had ~1 cm cut off of the open

end before being loaded into a 0.25 mL Cassou artificial insemination gun. A plastic outer protective sheath was placed over the barrel of the Cassou gun after loading. Using rectally guided assistance, the tip of the gun was passed into the vagina and as it approached the external cervical os, the tip was pushed through the outer sheath. Then, the tip of the Cassou gun was passed transcervically into the uterus and directed into the uterine horn ipsilateral to the ovary with a corpus luteum, where the embryo was deposited. Pregnancy evaluation was performed by transrectal ultrasonography 45 days after embryo transfer.

- **Statistical analysis**

The Chi-square test was used to determine any differences among semen treatments or somatic cells isolated from the two types of ewes. Statistical analysis was performed using the Graphpad InStat (version 3). A value of  $P < 0.05$  was considered to be significantly different for the study.

## **5.3 Results**

- **Isolation, culture and cryopreservation of somatic cells from semen**

GCN ram semen: Initial cell adhesion (attached) to the collagen-coated culture surface was observed in fresh ( $n=2$ ), cooled ( $n=3$ ) and slow-freezing ( $n=1$ ) replicates (semen treatment groups), but not from the fast-freezing treatment (Table 5.1). Some attached cells from fresh and cooled semen divided and formed proliferating colonies ( $n=1$  and  $n=3$ , respectively) that subsequently produced a monolayer covering about  $1 \text{ cm}^2$  of the surface, while attached cells from the slow-freezing treatment did not continue proliferation.

Somatic cells isolated from ram semen all had a similar epithelial-like morphological appearance (Figure 5.2) that was comparable with that of ram cells isolated from fresh and cooled semen during preliminary studies (unpublished earlier data). However, immunohistochemical detection of cell-associated proteins and DNA microsatellite analysis could not be performed, because the cells did not proliferate after the first passage (P1). Statistical analysis

Table 5.1. Stages that somatic cells isolated from fresh, cooled and frozen-thawed ram and eland semen reached during culture.

Species	Treatment	n <sup>a</sup>	Attach. <sup>b*</sup> (%)	Prolif. <sup>c*</sup> (%)	Pass. <sup>d*</sup> (%)	P1 Prolif. <sup>e*</sup> (%)
GCN	Fresh	7	2 (29)	1 (50)	1 (100)	0 (0)
	Cooled	7	3 (43)	3 (100)	1 (33)	0 (0)
	-1°C/minute	7	1 (14)	0 (0)	0 (0)	0 (0)
	-10°C/minute	7	0 (0)	0 (0)	0 (0)	0 (0)
Eland	Fresh	2	2 (100)	2 (100)	2 (100)	2 (100)

Attach., Prolif., Pass., and P1 Prolif. indicates attachment, proliferation, passage and first-passage proliferation as stages that were reached by each cell culture attempt. Percentages (%) shown in each column are calculated as a percentage of the preceding column.

<sup>a</sup> Total number of replicates (ejaculates) from two GCN rams and one eland bull.

<sup>b</sup> Number of replicates in which cells attached.

<sup>c</sup> Number of replicates in which attached cells proliferated.

<sup>d</sup> Number of replicates in which primary culture reached 1 cm and were passaged.

<sup>e</sup> Number of replicates in which cells proliferated after first passage.

\* Morphologically, all cells were epithelial-like.



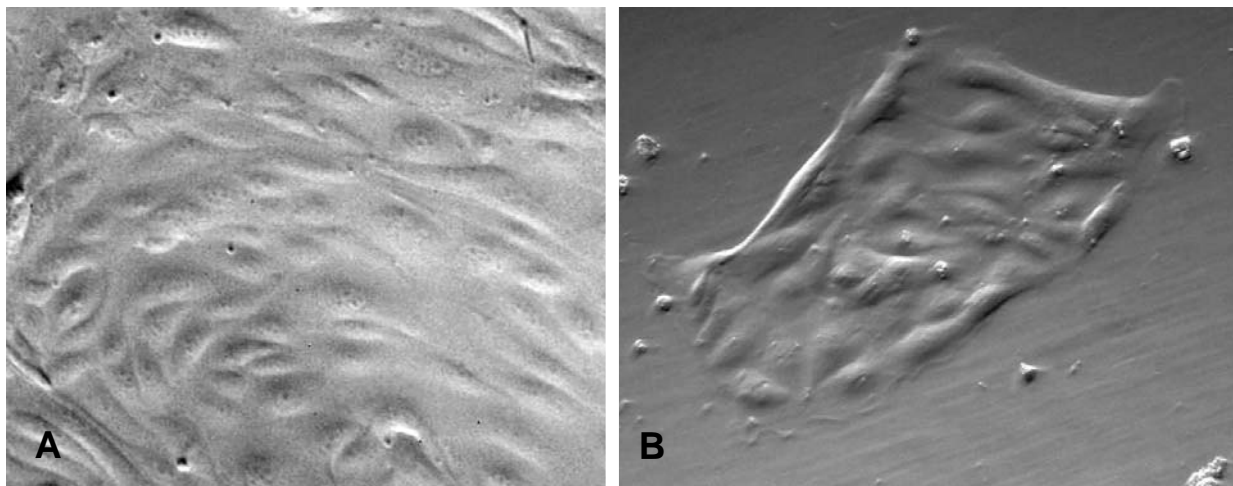


Figure 5.2 Epithelial cells isolated from GCN ram semen that were characterized during preliminary trials (A) and similar epithelial-like morphology of cells isolated from GCN ram semen during the current experiment (B).

did not indicate any significant differences between treatment groups for cell attachment, proliferation, passage or P1 proliferation.

Eland semen: Single cells derived from fresh semen (n=2) were able to attach, divide and proliferate until forming a monolayer colony covering ~1 cm<sup>2</sup> of the surface of the collagen-coated culture dish. These cells were successfully subpassaged and frozen at the second passage. Immunohistochemical detection of cytokeratin indicated that these eland cell lines were epithelial cells (Figure 5.3).

All 14 cattle-derived microsatellites amplified in DNA from the eland. Only two markers were polymorphic in the eland semen donor, however, the genotypes for the DNA sample from the eland blood and epithelial cells isolated from the semen were identical. Five markers showed variation in the second eland and in total, seven cattle-derived markers were effective in showing variation between the different eland.

▪ **Isolation, culture and cryopreservation of somatic cells isolated from fresh milk**

In this experiment, somatic cell colonies from 24 of 26 (93.0%) fresh milk samples collected from the GCN ewes and crossbreed GCN X Suffolk ewes were isolated and established. From these colonies, 42% (10/24) divided, proliferated and survived after passage 1, when they were cryopreserved (Table 5.2). No differences in cell isolation and cell division were found between purebred and crossbred ewes.

Immunohistochemical analysis of the somatic cells indicated that cell lines established from milk were either fibroblast, epithelial, or a mixture of the two cell types (Figure 5.4). Microsatellite analysis also indicated that these somatic cells were derived from the milk donors because they were genetically identical to fibroblast cells obtained from skin biopsies of the same sheep milk donors.

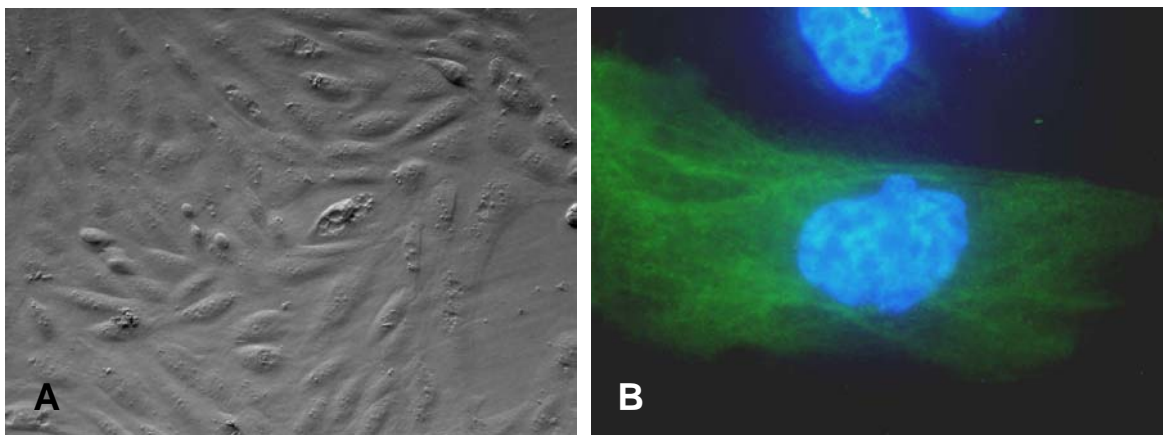


Figure 5.3. Epithelial-like cells isolated from eland semen (Hoffman modulation microscopy) (A) and eland semen-derived epithelial cells labeled with cytokeratin-FITC and Hoechst 33342 (epifluorescence) (B).

Table 5.2. Somatic cell attachment and proliferation from fresh GCN and GCN X Suffolk crossbred sheep milk.

Breed type	n <sup>a</sup>	Milk samples <sup>b</sup>	Cells attached <sup>c</sup> (%)	Proliferated <sup>d</sup> (%)
GCN	5	6*	5 (83)	3 (60)
GCN x Suffolk	10	20	19 (95)	7 (37)
Total	15	26	24 (92)	10 (38)

Data presents number of milk samples from which attachment or proliferation were reached from the total number of milk samples collected.

Percentages (%) shown in each column are calculated as a percentage of the number in the preceding column.

\* Four of 10 GCN milk samples were contaminated and omitted from the study.

<sup>a</sup> Total number of animals in each breed group.

<sup>b</sup> Total number of milk samples collected in each breed group.

<sup>c</sup> Number of samples from which cells attached in culture.

<sup>d</sup> Number of samples from which cells proliferated in culture.

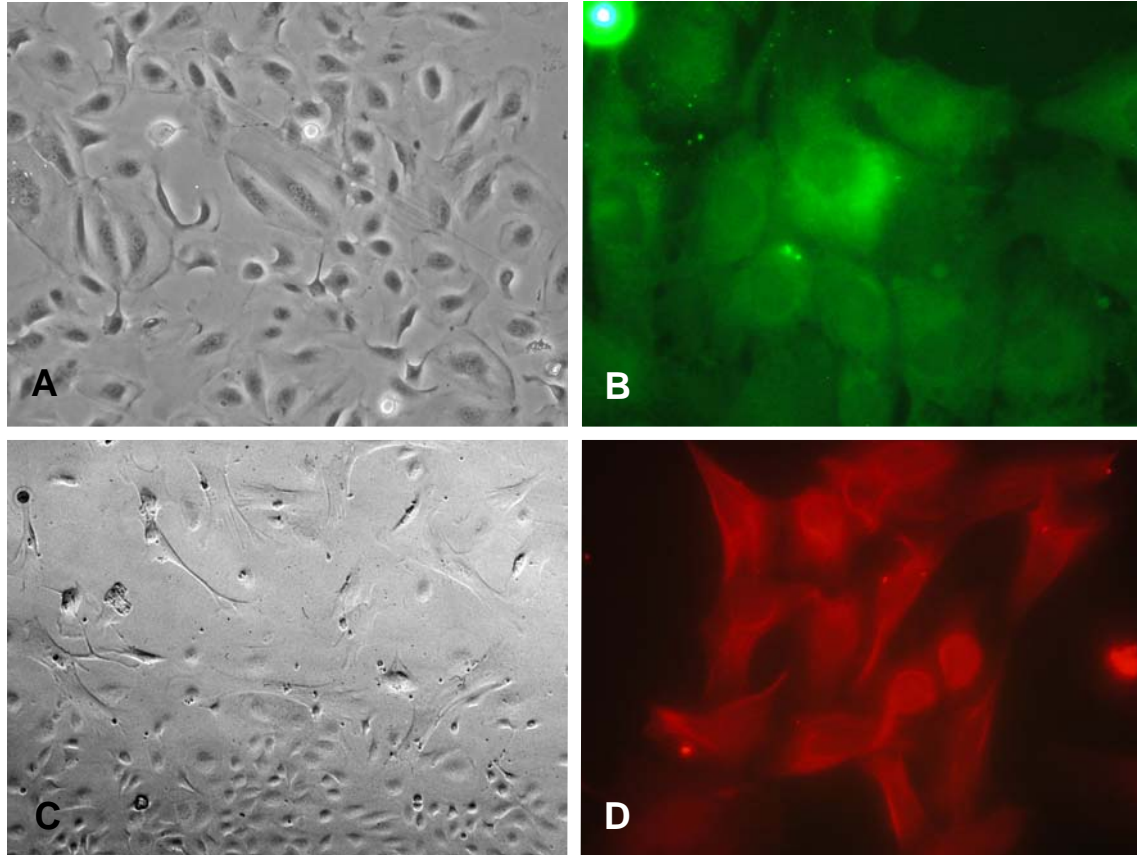


Figure 5.4. Epithelial (A + B) cells and fibroblast cells (C + D) isolated from sheep milk. Panel A and B show epithelial cells under phase contrast (A) and under epifluorescence after labeling with cytochrome-FITC (B). Fibroblast cells are observed adjacent to an epithelial colony in the same culture dish observed under Hoffman modulation contrast optics (C) and under epifluorescence after labeling with vimentin-TRITC (D).

#### ▪ Nuclear transfer and embryo transfer of cloned eland embryos into eland recipients

Eland oocytes were mechanically enucleated and electrically fused to frozen-thawed eland epithelial cells. A total of 17 couplets fused, with 12 (71%), 6 (35%) and 2 (12%) of the embryos cleaving and developing to the morula or blastocyst stages, respectively (Table 5.3).

A total of three morulae (day 7) and one blastocyst (day 7) stage cloned eland embryos were transferred into each of two eland recipients. No pregnancies were detected at transrectal ultrasonography on day 45 after embryo transfer.

### 5.4 Discussion

In this study we successfully isolated somatic cells from milk and ejaculated semen. To our knowledge, this is the first report of somatic cell isolation from semen for *in vitro* culture. Although we showed that cell isolation from semen is possible, cell propagation rates were low. Cells isolated from fresh, cooled and slow-frozen ram semen samples attached to the surface of the tissue culture dish, while cells isolated from fast-frozen semen did not attach. These results are similar to those observed in our preliminary studies (unpublished data), in which cells were successfully isolated from fresh and cooled samples, but not from conventionally cryopreserved semen. It is, therefore, possible that the cryopreservation method that was used for the ram semen might be detrimental to somatic cells.

Among the variables affecting cell survival during cryopreservation are the rates at which the cells are cooled and warmed and the type and concentration of cryoprotectant used (Ashwood-Smith, 1980; Armitage, 1986). Usually, dimethyl sulfoxide (DMSO) is the most common cryoprotectant used for freezing epithelial cells (Freshney and Freshney, 2002) and the freezing medium is commonly supplemented with serum (10 to 15%). In our study, cryopreserved ram semen was in a medium containing glycerol (5%) as cryoprotectant and supplemented with egg yolk (15%), which may not be the optimal medium for freezing somatic cells. Previous reports have shown that somatic cells from tissues and cell cultures can be

Table 5.3. Embryo development following nuclear transfer of eland epithelial-like cells isolated from semen into enucleated Common eland oocytes.

Eland recipient ID	Fused couplets (n)	Cleaved (%)	Morulae (%)	Blastocysts (%)	Embryos transferred (%)
123	10	6 (60)	3 (30)	1 (10)	4 (40)
140	7	6 (86)	3 (43)	1 (14)	4 (57)
Total	17	12 (71)	6 (35)	2 (12)	8 (47)

Percentages (%) are calculated as a percentage of the number of couplets produced.

frozen with glycerol as cryoprotectant (Luyet and Gonzales, 1952; Mazur et al., 1972; Ashwood-Smith and Lough, 1975). Chick epithelial cells and calf kidney epithelium have survived after being frozen in the presence of 5 to 10% of glycerol (Pomerat and Moorhead, 1956; Vieuchange, 1958). Although a slightly higher cell survival rate has been found after freezing hamster cells with DMSO when compared with those frozen with glycerol (Mazur et al., 1972; Ashwood-Smith et al., 1975), both DMSO and glycerol are widely used, effective cryoprotectants for cryopreservation of cell lines (Freshney, 1983a). Differences in cell survival are believed to be due to differences in toxicity, rather than cryoprotective capacity (Smith, 1961b; Ashwood-Smith, 1980). Therefore, it is unlikely that the use of glycerol as a cryoprotectant was solely responsible for our failure to isolate live epithelial cells from fast-frozen semen.

A more likely explanation may be the differences in the cooling rates usually used for freezing semen and somatic cells. Cooling rates for somatic cells are in the range of 1 to 5°C/minute and are followed by rapid thawing rates of 150 to 200°C/minute (Ashwood-Smith, 1980). Freezing semen in straws usually requires higher average cooling rates, although a nonlinear cooling curve is often used either by the heat-load inserted with the straws into the pre-cooled freezing chamber, or by multi-step freezing procedures (Smith, 1961c; Polge, 1980; Muldrew et al., 2004). We used a constant cooling rate of -10°C/minute to freeze semen in 0.5 mL straws. This conventional technique was chosen to benefit the spermatozoa and might not be optimal for somatic cell survival, since much variation exists between spermatozoa and somatic cells in their response to freezing (Armitage, 1986).

Intracellular ice formation is one of the primary causes of freeze damage during cell cryopreservation and this is normally prevented by achieving partial dehydration, thereby reducing the freezing point of the cell interior. Accordingly, it is important that a cell loses a sufficient amount of water by osmosis during the cooling process to survive cryopreservation (Mazur, 1963). At low cooling rates, cells have enough time to equilibrate with the increasing



extracellular osmolarity, allowing intracellular shrinkage and preventing ice formation. During higher cooling rates, however, the cells cannot lose water quickly enough to maintain osmotic equilibrium and supercooling and freezing occurs before cells are dehydrated sufficiently to prevent damage (Armitage, 1986; Muldrew et al., 2004). Since water-permeability varies greatly with cell type and, especially, cell size, it is probable that epithelial cells will behave differently from spermatozoa during the same cooling and thawing cycle, even in the presence of the same cryoprotectant. Therefore, even if the technique that we used to freeze the semen is optimum for sperm survival, it does not appear to support high survival rates for somatic cells.

Optimum thawing rates are dependant on the freezing rate (Armitage, 1986; Mazur, 2004). Therefore, in the present study, an alternative possibility is that the cells may have survived rapid cooling, but the thawing rate, while effective for spermatozoa, was not optimal for somatic cell survival. It is also important to note that the optimum cooling curve is, to a great extent, dependant on the type and concentration of cryoprotectant used (Armitage, 1986). Even if the conventional slow-freezing method was used for somatic cells, the difference in type and concentration of cryoprotectant may have changed the optimal cooling curve, possibly explaining the low results in the slow-freezing treatment.

Furthermore, cryopreservation may sensitize cells to other stressors, such as dilution of the cryopreservation medium and centrifugation (Lovelock, 1953; Farrant and Morris, 1972), or even to unfavorable culture conditions that cells would normally withstand. The procedure used during the present experiment included dilution and three washing steps to ensure that the cryoprotectant was removed and to minimize contamination. Likely, some epithelial cells survived the freeze-thaw cycle, but were damaged during post-thaw processing. Likewise, a percentage of ram cells possibly survived both cryopreservation and post-thaw processing, but due to suboptimum culture conditions, were unable to attach to the substrate. Therefore, further studies should be focused on optimizing the culture conditions specifically for semen-derived epithelial cells.

In preliminary experiments somatic cells isolated from eland and sable antelope semen were identified as epithelial cells. The exact origin of the somatic cells is not known, but it is possibly a mixture of tubule epithelial cells that were sloughed off from the accessory glands, seminiferous tubes and the vas deferens. In the present study, the morphology of the ram cells was similar in appearance to that of the previously identified ram, eland and sable antelope epithelial cells (Figures 5.2 and 5.3).

A multitude of factors affect growth of epithelial cells *in vitro*. Epithelial cells require more complex culture systems than fibroblast cells, such as substrate modification, additional medium supplementation and different dissociation techniques (Freshney and Freshney, 2002). In our experiments, collagen-coated dishes were used to support growth *in vitro*. Although attachment and some proliferation were observed, suboptimal culture conditions may have contributed to the low cell proliferation. Another aspect that may be affecting the initial attachment is the presence of motile spermatozoa. Sperm motility was maintained for over 24 hours after initiation of culture and this may have physically prevented cells from attaching to the culture surface. Therefore, further experiments should be conducted to improve the culture system and enhance separation of somatic cells from spermatozoa before culture. This would enhance cell attachment to the substrate and support proliferation of epithelial cells.

Results from the microsatellite analysis on eland epithelial cells corresponded to results from preliminary experiments with ram semen, where semen-derived ram epithelial cells were genotypically identical to fibroblast cells from the semen donor.

We found that success rates in isolating and propagating somatic cells from milk far exceeded results obtained with semen. One possible explanation is that the volume of milk used was typically between 30 and 40 mL, whereas, the average volume of the ram ejaculate was between 1 and 2 mL. Therefore, we expected that the starting cell numbers harvested from milk should be larger than that of cells from semen. Furthermore, epithelial cells, fibroblast cells and a mixture of cell types were isolated from milk. The presence of fibroblast cells in several of the

isolated cultures and the uniform fibroblast cell cultures that were occasionally obtained should increase the likelihood of obtaining somatic cell attachment and proliferation from milk since the culture system is already optimized for this cell type. In mixed cell cultures, the fibroblast cells may actually serve as feeder cells for the epithelial cells.

One successful method that is widely used for enhancing epithelial culture conditions is co-culture with inactivated fibroblast cells (Macpherson et al., 1971). In addition to serving as an attachment substrate and a source of continuously secreted growth factor, feeder cells may inactivate the inhibitory action of serum-derived Transforming Growth Factor-Beta (TGF- $\beta$ ) on epithelial cells (Tucker et al., 1984; Moses et al., 1985; Masui et al., 1986). Therefore, this effect could be partially responsible for the higher success rates in milk cell proliferation. When there are low cell numbers and only fibroblast cells attach, a low concentration of cells at initiation of culture may be detrimental for fibroblast proliferation. In contrast, our results suggest that cultures with small numbers of epithelial cells at onset of culture can proliferate much further than those with low numbers of fibroblasts.

Highly specific culture procedures have been described for human mammary epithelial cells (Stampfer et al., 2002) and incorporating these techniques should improve results. We demonstrated that milk from nondairy animals could be a source of somatic cells for *in vitro* culture, but ideally, each primary culture cell type should be identified and the culture system modified as required.

The DNA microsatellite analysis confirmed that cells from semen and milk were from the respective donors, which implies that cells could be used for innumerable genetic studies and be incorporated in genetic resource banks. Additionally, bovine microsatellite markers proved to be effective in the eland. Ultimately, isolated cells could serve as karyoplast donors during nuclear transfer procedures. Our NT results indicated that eland epithelial cells isolated from semen could direct early embryonic development after transfer into eland cytoplasts.

Although two embryos developed to blastocysts *in vitro*, no pregnancies were established after the transfer of cloned eland blastocysts into eland recipients.

Culture methods have not been optimized for *in vitro*-produced eland embryos (Wirtu, 2004). To our knowledge, eland pregnancies have not been established following *in vitro* embryo production. Therefore, the inability to establish pregnancies could be attributed to suboptimal conditions that these embryos were exposed to prior to embryo transfer. Even in domestic cattle where extensive research has been ongoing for years and *in vitro* culture to the blastocyst stage has been relatively successful (~40%), success rates of producing live offspring following NT in most species (i.e., cows and sheep) still remains less than 4% (Wilmut et al., 2002). It is therefore not surprising that pregnancies were not established from this species of which still so little is known. Future research should focus on optimizing *in vitro* protocols specifically for eland embryo production and culture.

In conclusion, we have described a novel technique for obtaining somatic cells from semen and their subsequent use for *in vitro* cell culture. The isolation of somatic cells from cryopreserved semen could have valuable implications for critically endangered species. Potentially, a clone could be produced from a deceased but genetically valuable animal using cells obtained from frozen semen and subsequently, additional offspring produced by naturally breeding the resulting cloned offspring.

## CHAPTER 6

### ***IN VITRO* PROLIFERATION OF EPITHELIAL CELLS ON EMBRYONIC FIBROBLASTS AFTER ISOLATION FROM SEMEN BY GRADIENT CENTRIFUGATION**

#### **6.1 Introduction**

In addition to sperm cells, semen contains leucocytes and sloughed epithelial cells from the male reproductive ducts (Gorus and Pipeleers, 1981; Makler et al., 1998). These somatic cells are diploid and therefore, could be potentially used as karyoplast donors for nuclear transfer procedures (Wilmut et al., 1997; Lanza et al., 2000; Gómez et al., 2004). Epithelial cells have been successfully isolated from human semen (Phillips et al., 1978). Similarly, we isolated somatic cells from whole semen of rams (Nel-Themaat et al., 2004) and eland antelope (Unpublished, 2003). However, our experiment did not involved separation of epithelial cells before culture so that potentially valuable sperm cells were lost in the process.

Cell separation by density gradient centrifugation has been widely and effectively used for isolation of various types of cells. Examples in which Percoll was successfully used to isolate cells include: Leydig cells (Schumacher et al., 1978), various types of leucocytes (Ulmer and Flad, 1979; Gutierrez et al., 1979; Kurnick et al., 1979; Watt et al., 1979; Feucht et al., 1980; Enerback and Svensson, 1980; Hjorth et al., 1981), erythrocytes (Rennie et al., 1979; Bennett and Kay, 1981), epithelial cells (Yang et al., 1980; Carballada and Saling, 1997), endothelial cells (Bowman et al., 1981; Nees et al., 1981), spermatozoa (Gorus et al., 1981; Berger et al., 1985) and tumor (Bosslet et al., 1981) cells. Although Percoll is commonly used to separate motile from nonmotile spermatozoa and other seminal cells (Gorus et al., 1981; Makler et al., 1998; Berger et al., 1985), there is no indication that it has been used previously to harvest a specific somatic cell from semen.

In Chapter V, low proliferation rates of semen-isolated somatic cells were observed, and mainly caused by suboptimal *in vitro* culture conditions. Epithelial cells require specialized

culture conditions for optimum proliferation, as opposed to fibroblasts that are considered relatively easy to culture (Freshney and Freshney, 2002). Inactivated 3T3 mouse fibroblasts are frequently used in co-culture systems with many types of epithelial cells (Freshney, 2002). Cells of interest can be either plated directly on the feeder cells, or cultured in pre-condition medium that feeder cells were cultured in (Kabalin et al., 1989).

Therefore in this study an attempt was made to (1) isolate somatic cells from ram and eland semen while preserving viable spermatozoa by Percoll gradient separation and (2) to optimize *in vitro* culture conditions for epithelial cell proliferation.

## **6.2 Material and Methods**

### **▪ Experimental design**

During the first experiment, the four treatments were different Percoll fractions: (1) 0%, (2) 20%, (3) 50% and (4) 90%. For each treatment, the number of attempts in which cells (a) attached, (b) divided and (c) proliferated were recorded. A total of 24 replicates (collections) from two rams and four replicates from one eland bull were performed. No direct comparison was made between the two species.

In the next experiment, the three different co-culture treatments were: (1) on feeder, (2) insert and (3) collagen. For each treatment, we measured (a) attachment, (b) division, (c) proliferation and (d) proliferation after passage. A total of 20 replicates (ejaculates) from two rams and two replicates from one eland bull were performed. The cell lines obtained were then evaluated using two criteria (immunohistochemical detection of cytoplasmic proteins and morphology) to determine cell types obtained from the different co-culture groups.

Cryopreserved semen of four ram and four eland ejaculates were also cultured based on results from the co-culture experiment. Origin of the resulting cell lines were determined by DNA microsatellite analyses.

## ▪ **Animals**

Mature Gulf Coast Native (GCN) rams (n=2), from the Louisiana State University Agricultural Center Sheep Research Unit in Baton Rouge, Louisiana, and an eland bull (n=1) were housed at the Freeport-McMoRan Audubon Species Survival Center (FMA-SSC) near New Orleans, Louisiana. The two rams (a 2-year old and a 6-year old) with body condition scores of 4 and 3 (on a scale of 1 to 5), respectively, were fed once daily (Purina Show Chow lamb ration with bermudagrass hay always available). The 7-year old eland bull (~530 kg) was also fed once daily (Purina ADF-16 herbivore feed). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Audubon Center for Research of Endangered Species as required by the Health Research Extension Act of 1985 (Public Law 99-1580).

## ▪ **Chemical reagents**

Chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA), unless otherwise stated.

## ▪ **Semen collection and processing**

Rams were allowed to acclimate for 7 days after arrival and a “cleanout” ejaculation was performed before semen was collected for the experiment. Ram semen was collected by electroejaculation (Evans and Maxwell, 1987), while eland semen was collected by a combination of rectal massage and electroejaculation (Wirtu et al., 2002b). Two ejaculates were collected from each ram with 20 to 30 minutes of rest between ejaculates, with at least 3 days of rest between procedures. Two ejaculates were obtained from the eland male on each collection day, 2 weeks apart. The intercollection rest period was greater for the eland bull since the animal had to be sedated at each collection, which could be a health risk for the animal if performed at higher frequencies. Fresh semen samples were allowed to cool to 30°C before dilution with Salamon’s ram semen extender (1:1; v/v) containing 36.34 mg/mL of Tris {hydroxymethyl} aminomethane, 5 mg/mL of glucose, 19.9 mg/mL of citric acid (Mallinckrodt,

Hazelwood, MO, USA), 1,000 IU/L of penicillin-G and 1 mg/mL of streptomycin sulfate, without egg yolk and glycerol (Evans and Maxwell, 1987). Samples were allowed to cool to room temperature (~22°C) before further extension to a total volume of 10 mL. Extended semen was centrifuged for 10 minutes (500 x g), washed twice with Ca<sup>2+</sup> and Mg<sup>2+</sup>-free Dulbecco's phosphate-buffered saline (DPBS, Gibco, Grand Island, NY, USA) and the pellet resuspended in 2.5 mL of minimum essential medium alpha medium (α-MEM; Gibco) supplemented with 15% newborn calf serum (NCS), 1% MEM nonessential amino acids (NEAA), 5% penicillin-streptomycin (Pen/Strep; Cellgro, Herndon, VA, USA) and 250 µg/mL of gentamicin (Gibco).

#### ▪ Cell isolation by Percoll gradients

Gradient columns consisted of 2.5 mL each of 90% (P-90), 50% (P-50) and 20% (P-20) of Percoll (Fluka Chemie GmbH, Steinheim, Sweden) diluted in DPBS. Columns were layered in 15 mL conical tubes in the order listed above. Each washed semen sample (2.5 mL) was layered carefully on top of the Percoll gradient and centrifuged at 400 x g for 20 minutes. Then, each layer and interface band was collected by careful aspiration using a sterile Pasteur pipette. All fractions, including the supernatant containing 0% Percoll (P-0), and the pellet in the 90% fraction, were washed as described above and resuspended in α-MEM before plating on 35-mm collagen Type I-coated (10 µg/cm<sup>2</sup>) cell culture dishes.

After centrifugation, 20 µL from each fraction were diluted with an equal volume of Trypan Blue (TB) to determine the membrane integrity of somatic cells collected. Sperm motility was evaluated visually under light microscopy at 200X magnification by estimating the percentage of progressively motile cells in three different fields of view. Morphology was assessed visually at 400X magnification by determining the percentage of sperm cells that had apparent abnormalities (i.e., coiled or bent sperm tails). These evaluations, however, were only the basic estimations of spermatozoa quality, and no further assessment of sperm status was performed.



#### ▪ Culture of somatic cells with mouse feeder layers

Cultured 3T3 mouse fibroblast cells were inactivated by exposure to 30 µg/mL of mitomycin C in α-MEM for 90 minutes. Then, aliquots were resuspended in freezing medium consisting of α-MEM supplemented with 10% dimethyl sulfoxide (DMSO) and cooled at 1°C/minute to -80°C (Mr. Frosty; Nalgene, Rochester, NY, USA) before storage in liquid nitrogen (LN<sub>2</sub>). The day before the feeder layers were needed, cells were thawed and plated at 20,000 cells/cm<sup>2</sup> (1) in the first two wells of a collagen coated six-well insert companion culture plate (feeder layers), (2) in the second two wells on 1 µm pore size polyethylene terephthalate membrane culture inserts (inserts), while (3) the third two wells remained without feeder cells (collagen) (Figure 6.1). The P-20 layers of semen samples from the first or second ejaculates of the day were divided into three aliquots before plating on the first or second row of wells of prepared feeder plates, respectively. Culture dishes were placed in a humidified incubator at 38.5°C in 5% CO<sub>2</sub> in air. After 24 hours, wells were rinsed three times and cultured in α-MEM containing 1% penicillin/streptomycin solution for a final concentration of 100 IU of penicillin and 0.1 mg/mL of streptomycin. Culture medium was changed every 3 to 4 days and dishes were searched for cell attachment (A), division (D) and proliferation (P). When colonies reached ~0.5 cm<sup>2</sup>, subpassage was performed by incubation in Versene 1:5000 (Gibco) for 10 minutes, followed by incubation in 0.125% trypsin in DPBS until cells were detached from the culture surface. When confluency (P1) was reached, cells were cryopreserved and stored in LN<sub>2</sub>.

#### ▪ Isolation of somatic cells from cryopreserved semen

Semen samples that were previously cryopreserved in independent projects were used. Five straws (total volume = 2.5 mL) from single ejaculates of four rams that were frozen in extender containing egg yolk, and four ejaculates from one eland bull that were previously cryopreserved in either milk or egg yolk extender, were thawed and processed on Percoll gradients as previously. Cells from the P-20 layer were plated on collagen in the presence of

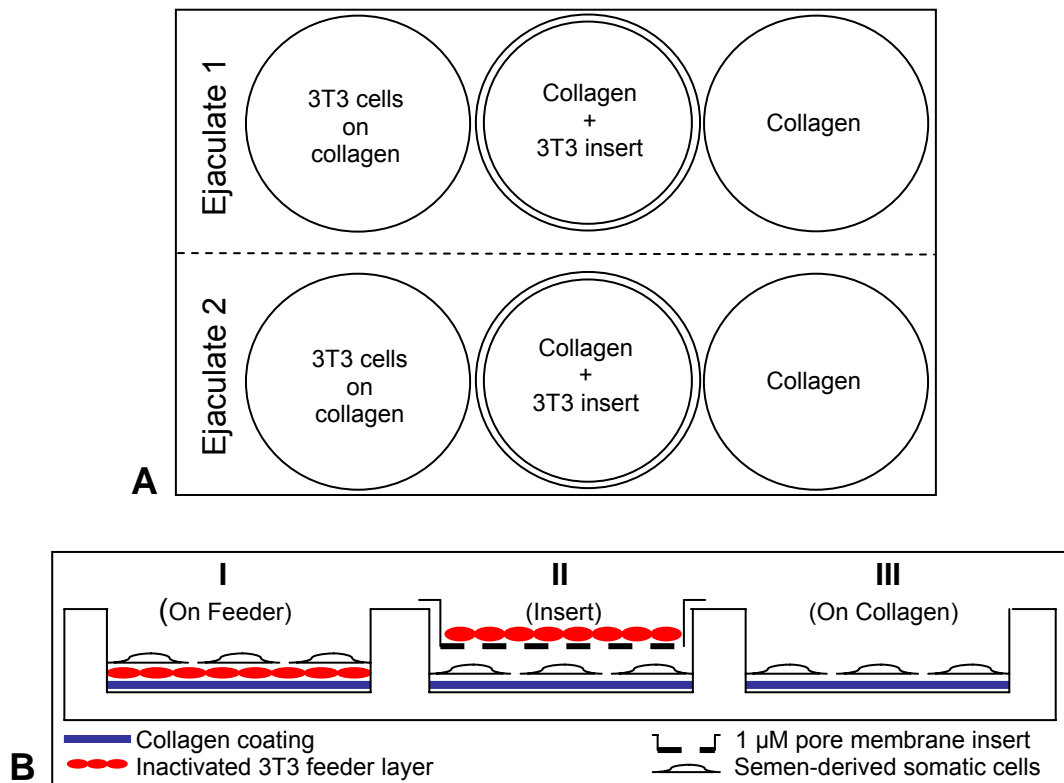


Figure 6.1. Distribution of collagen-coated 6-well insert companion culture plates used to co-culture 3T3 mouse embryonic fibroblasts and semen-isolated cells from P-20 fractions after Percoll separation (top view) (A) and a lateral view of a six-well culture plate with three treatments of co-culture (B).

3T3 inserts. Cell attachment (A) and proliferation (P) were assessed as previously described, and cell morphology was assessed and recorded.

#### ▪ **Immunohistochemical detection of cell types**

Cells for immunohistochemical characterization were plated on collagen-coated culture chamber slides (Biocoat, Becton Dickinson & Company, Franklin Lakes, NJ, USA) at first passage and cultured until several colonies were visible. Cells were then fixed for at least 12 hours in paraformaldehyde (PF, 3.7% in DPBS). Slides were sealed with Parafilm and stored at 4°C until further staining. Epithelial cells were identified by the detection of cytokeratin as previously described (Sun and Green, 1978) with only slight modifications. Briefly, cells were permeabilized for 10 minutes in 1% Triton-X diluted in PBS (PBS-T), rinsed twice in 0.1% Tween 20 in PBS (PBS-T20) and stabilized with 0.1 M glycine solution. Then, cells were either incubated in PBS-T20 containing primary guinea pig anti-keratin (1:20), or in primary mouse anti-vimentin (1:200) for at least 2 hours on a platform shaker, rinsed three times in PBS-T20 and incubated with secondary anti-guinea pig IgG conjugated either with Alexa Fluor® 594 for keratin (1:200, Molecular Probes, Inc., Eugene, OR, USA), or with anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) for vimentin (1:60) for 1 hour.

Positive controls were labeled with mouse anti- $\beta$ -tubulin and anti-mouse IgG TRITC conjugate, whereas, negative controls were incubated with secondary anti-mouse or anti-guinea pig conjugated antibodies. Cells were rinsed and incubated in 20  $\mu$ g/mL of Hoechst 33342 to stain nuclei, before three additional rinses in PBS-T20. Finally, chambers were removed, slides were covered with FluoroGuard™ Antifade reagent (Bio-Rad Laboratories, Hercules, CA, USA) and a cover slip was mounted before examination by epifluorescence microscopy.

#### ▪ **Statistical analysis**

Fisher's Exact test was used to determine any differences of attachment, division, proliferation and cell proliferation at P-1 among Percoll fractions and feeder layer treatments. P-values smaller than 0.05 were considered significant in this study. Statistical analyses were

performed using the Graphpad Instate software (Graphpad Software, Inc., San Diego, CA, USA).

## 6.3 Results

### ▪ Cell isolation by Percoll gradients

After centrifugation of semen through Percoll gradients, three interface bands were obtained within each fraction, and a pellet was formed at the bottom of the tube (Figure 6.2). Different types of somatic cells and spermatozoa were collected at each interface band (Table 6.1). The P-0 fraction contained mostly flat, angular shaped cells that frequently appeared as sheets and stained blue with TP (dead cells) (Figure 6.3 A). In the P-20 fraction, cells appeared smoother, three-dimensional and a larger portion of the cells remained unstained by TP (intact cell membranes) (Figure 6.3 B). Few spermatozoa were found in the P-0 and P-20 fractions. In the P-50 fraction, somatic cells were smaller, elongated and stained blue with TP (Figure 6.3 C). Most spermatozoa in this fraction were nonmotile and had coiled tails (Figure 6.4 A). The P-90 fraction contained a high concentration of progressive motile spermatozoa (Figure 6.4 B) and no somatic cells were observed (Figure 6.3 D).

Motile spermatozoa from the P-90 fraction placed in culture, readily attached to the collagen-coated surface of the culture dish and appeared to swim synchronously in the direction of attachment, causing severe currents and swirling movement in the culture medium. Nonmotile spermatozoa or debris present in the P-90 fraction were rapidly flushed around in the culture dish as the currents increased, while more spermatozoa attached to the culture surface. Some spermatozoa remained attached to the culture dish surface after motility ceased, while other spermatozoa remained motile until medium was changed 24 hours later.

Table 6.2 is a summary of the total number of ram and eland culture attempts from which somatic cells attached, divided and proliferated from each Percoll fraction after plating on collagen-coated cell culture dishes (Figure 6.5). Typically, the number of colonies obtained

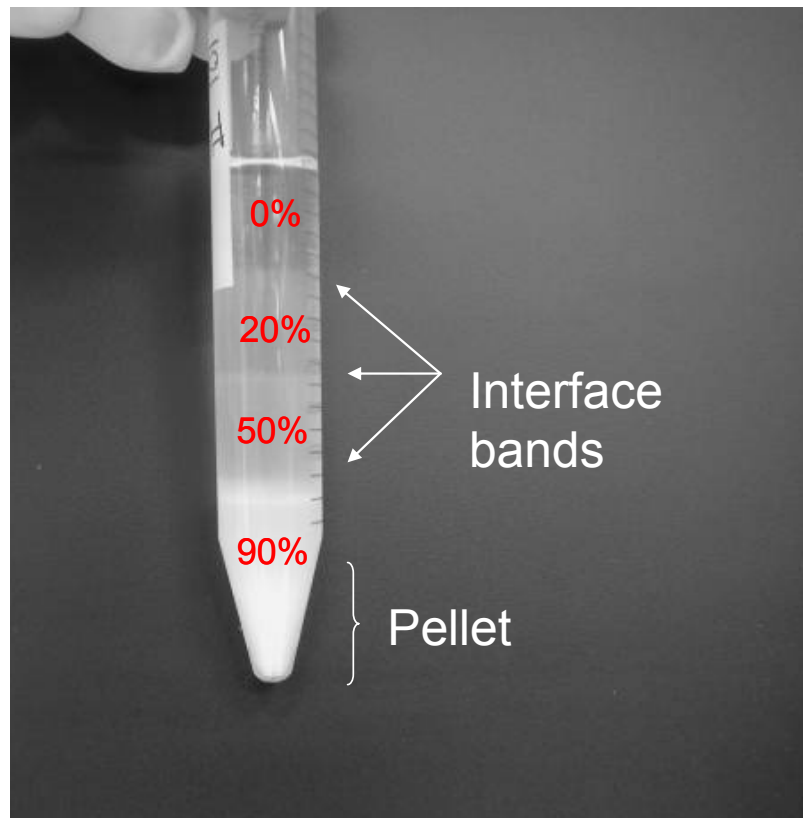


Figure 6.2. Interface bands obtained after centrifugation of a washed GCN ram semen sample through a Percoll gradient column.

Table 6.1. Somatic cells and spermatozoa isolated from Percoll fractions, with corresponding figure references in parenthesis.

Fraction	Somatic cells isolated	Spermatozoa isolated
P-0	Flat, angular and mostly nonintact membranes (Figure 6.3 A)	Negligible
P-20	Round, more intact membranes (Figure 6.3 B)	Negligible
P-50	Smaller, elongated, more nonintact membranes (Figure 6.3 C)	Abnormal, nonmotile and non-progressively motile (Figure 6.4 A)
P-90	Cells not visible due to high spermatozoa concentration (Figure 6.3 D)	Progressively motile and normal morphology (Figure 6.4 B)

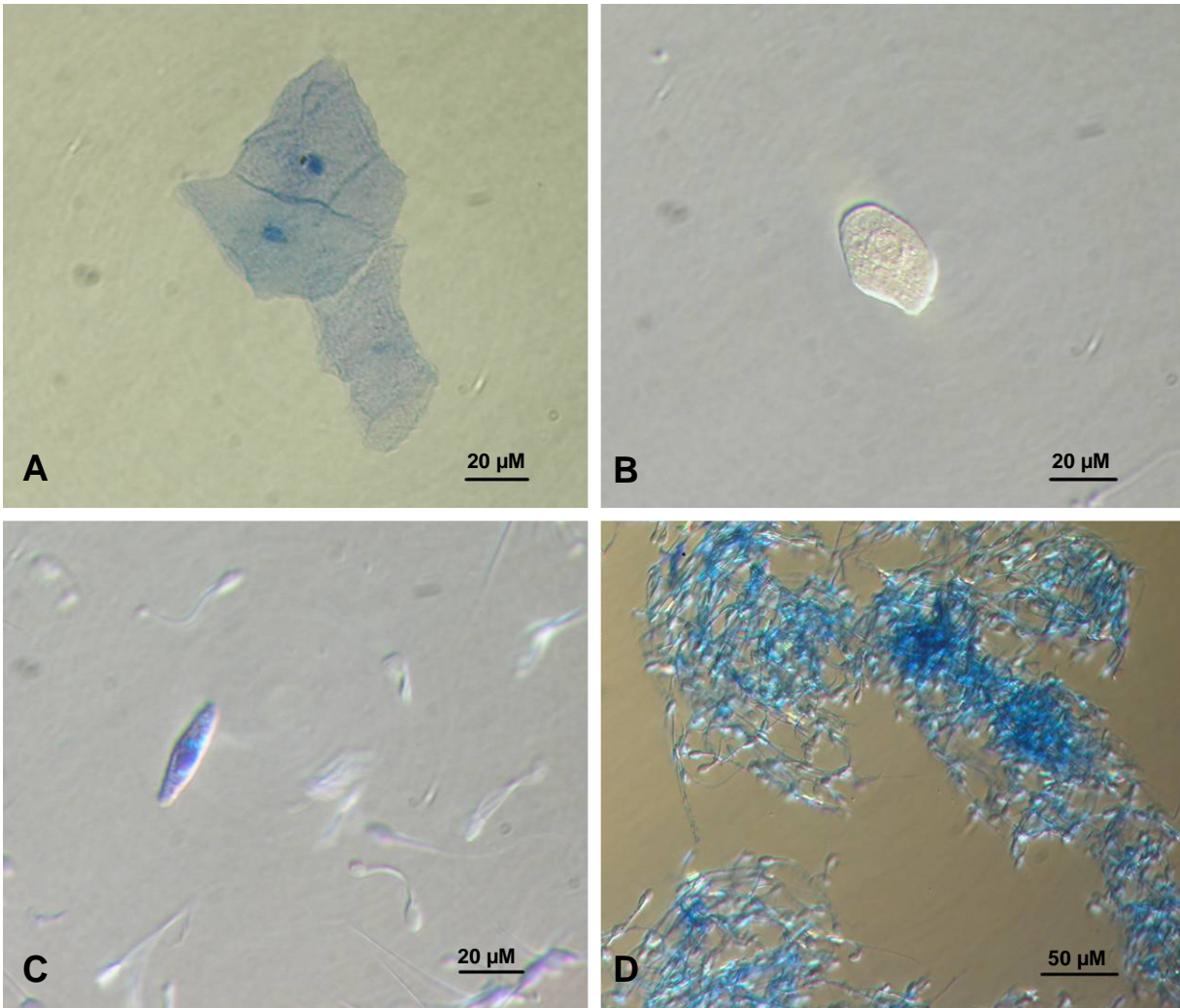


Figure 6.3. Ram somatic cells isolated from different Percoll gradients. P-0 fraction (A): flat, angular shaped somatic cells and stained blue with Trypan Blue (TB). P-20 fraction (B): somatic cells appeared smoother, three-dimensional and a larger portion of the cells remained unstained by TB. P-50 fraction (C): cells were smaller, elongated and stained blue with TP. P-90 fraction (D): no cells observed and high concentration of progressive motile spermatozoa was obtained.

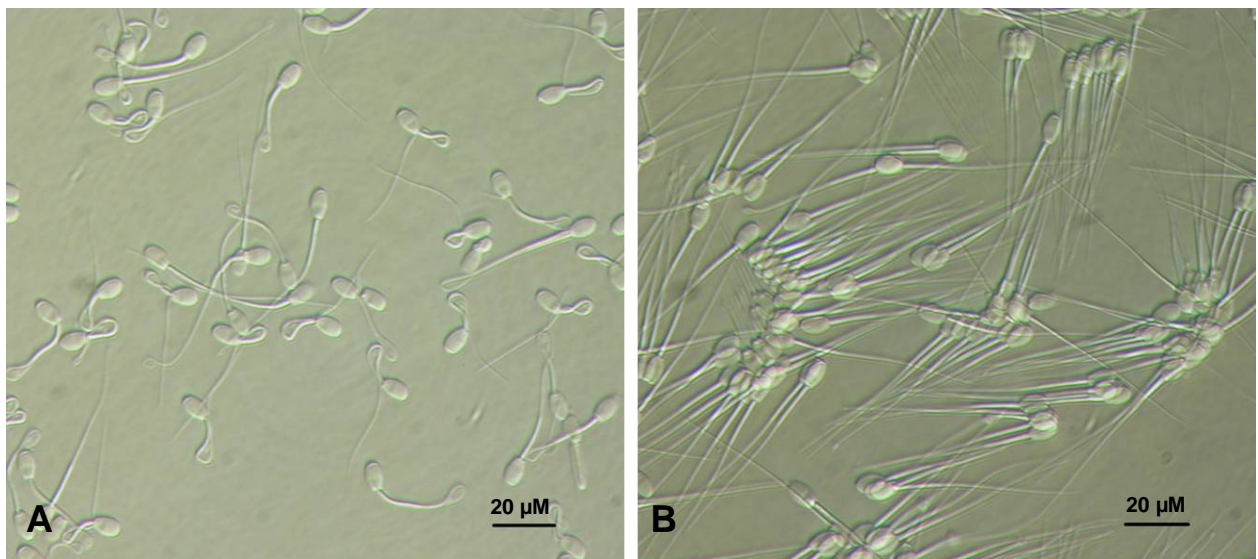


Figure 6.4. Ram spermatozoa collected from P-50 fraction were nonmotile and had coiled tails (A). Progressive motile spermatozoa were collected from P-90 fraction (B).



Table 6.2. Attachment (A), division (D) and proliferation (P) of ram and eland somatic cells isolated from semen by Percoll gradients and cultured on collagen coated cell culture dishes.

Species (no. animals)	n <sup>a</sup>	Somatic cell	P-0 (%)	P-20 (%)	P-50 (%)	P-90 (%)
Ram (2)	24	A	1 (4)	10 (42)	5 (21)	0
		D*	1 (100)	5 (50)	1 (4)	-
		P**	0	3 (30)	0	-
Eland (1)	4	A	1 (25)	3 (75)	0	0
		D*	1 (100)	2 (67)	-	-
		P**	1(100)	2 (67)	-	-
Total	28	A	2 (8)	12 (46)	5 (18)	0
		D*	2 (100)	7 (58)	1 (4)	-
		P**	1 (50)	5 (42)	0	-

<sup>a</sup> Number of ejaculates that were processed.

\* Ratio of attached cell samples that divided *in vitro*.

\*\* Ratio of attached cell samples that proliferated.

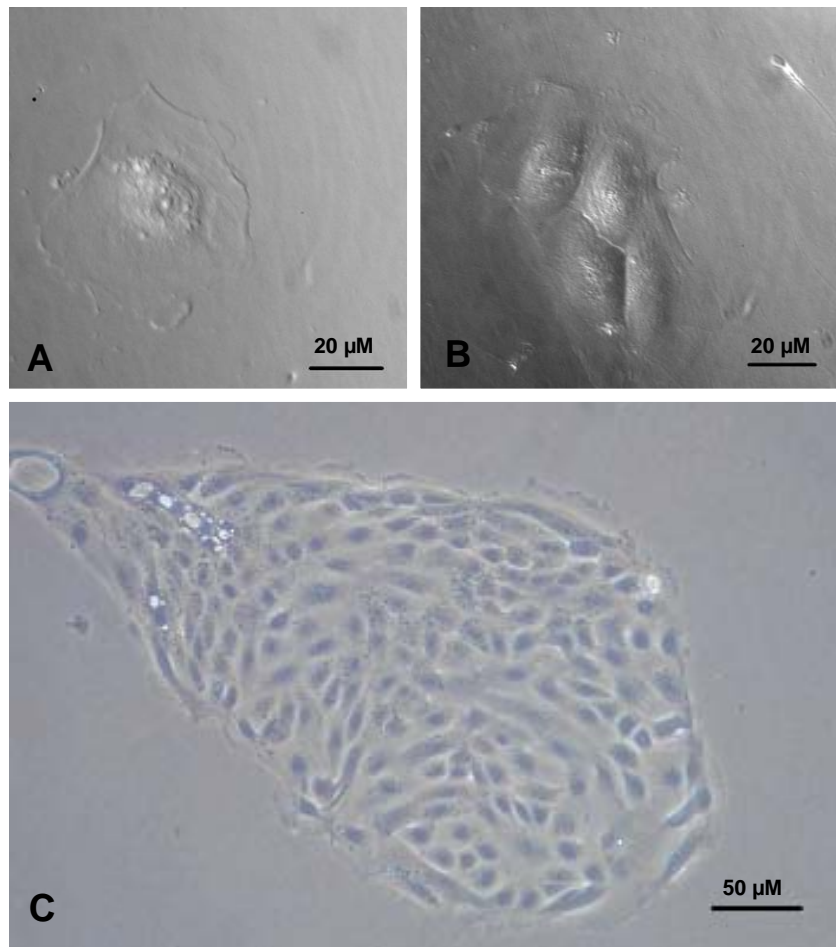


Figure 6.5. Semen-derived somatic ram cells attached (A), dividing (B) and proliferating (C) on collagen-coated surfaces under Hoffman modulation microscopy.

per ejaculate ranged between zero and five, although initial attachment of cells ranged between zero and 10 (estimation). For the rams, the percentage of somatic cell attachment to the culture dish (42%) from the P-20 fraction was similar to the percentage of attachment from the P-50 fraction (21%), but significantly greater than that of the P-0 or P-90 fraction (4% and 0% respectively) ( $P < 0.01$ ). Of cultures with attached cells, 100% (P-0), 50% (P-20) and 20% (P-50) showed cell division, while only cells from the P-20 fraction proliferated and formed colonies (30% of attempts in which cells attached). The majority of the ejaculates that resulted in cell attachment were from the younger ram (70%, data not shown), although division and proliferation did not appear to be influenced by age.

For eland, somatic cells isolated from the P-0 (25%) and P-20 (75%) fractions resulted in cell attachment (25% and 75%), division (100% and 67%) and proliferation (100% and 67%) respectively, whereas, no cell attachment was observed from the P-50 and P-90 fractions.

#### ▪ **Culture of somatic cells with feeder layers**

The rate of attachment and division of somatic cells isolated from the P-20 fraction were assessed for the insert and collagen treatments and not for the feeder cells treatment. In the feeder cells treatment, it was not possible to distinguish single cells and small colonies of epithelial cells among feeder mouse fibroblast cells. Therefore, the feeder cells treatment was evaluated only for proliferation and passages. The number of cells that attached per culture well ranged between zero and five (zero to 15 per ejaculate). On average, ejaculates from the younger animal yielded approximately double the amount of cell attachment of that from the older ram across all treatments (data not shown). In fact, proliferating cell lines were obtained from every ejaculate from the younger animal, whereas, only six of the 10 ejaculates from the older ram resulted in cell lines. However, division and proliferation was not affected by the ram age. There was also no change over time in the number of attempts in which cells attached.

For rams, no statistical differences were detected for attachment of somatic cells cultured on feeder inserts (80%) or collagen (70%), but higher percentage of cell division (80%)

was observed on the feeder inserts compared with cells on collagen (35%) ( $P < 0.01$ ) (Table 6.3). After division and first passage (P1), cells continued to proliferate at similar rates on the insert and feeder cells treatment (60% and 70%, respectively) and at higher rates than in the collagen treatment (10% and 5%, respectively). Cells that proliferated and continued dividing after P-1 were small colonies of epithelial-like cells that pushed fibroblast cells aside as the colonies expanded (Figure 6.6).

Cell attachment and division occurred in all treatments for both replicates for the eland. Cell colonies that were established continued to proliferate in all treatments. Most cells passaged at P1 continued proliferating, while only 50% of the collagen group proliferated after P1 (Table 6.3). Morphology of eland cell colonies (Figure 6.7) was comparable with that of ram cell colonies (Figure 6.6 C). For cells from both species, established colonies cultured without feeder cells sometimes ceased proliferation, developed a flat enlarged morphology and became senescent (Figure 6.8). Epithelial-like cells of various morphologies and sizes were observed among replicates and treatments, as well as within some replicates.

#### ▪ Isolation of somatic cells from cryopreserved semen

Somatic cells attached in all four culture attempts from both the ram and eland semen samples (Table 6.4). For the ram semen, cells proliferated in all four samples, whereas, only in two of the four eland culture attempts cells proliferated. Of these, only one cell line from ram (Figure 6.9 A) and one from eland (Figure 6.9 B) semen showed epithelial morphology, while the rest of the ram and eland semen cultures were fibroblast-like. Results from DNA microsatellite analysis indicated that all the fibroblast-like cells from both cryopreserved ram and eland semen were of murine origin and therefore 3T3 mouse fibroblast feeder cells. The epithelial-like cells from ram semen tested positive with sheep markers, whereas, the epithelial-like cells from eland semen tested negative with mouse markers, positive with bovine markers

Table 6.3. Isolation and proliferation of somatic cells isolated from P-20 Percoll fractions and cultured on feeder cells, with feeder inserts and on collagen.

Species (no. animals)	n <sup>1</sup>	Cell stage	Feeder cells	Feeder insert	Collagen
Ram (2)	20	Attached	N/A*	16 (80)	14 (70)
		Divided	N/A*	16 (80) <sup>a</sup>	7 (35) <sup>b</sup>
		Proliferation	12 (60) <sup>a</sup>	14 (70) <sup>a</sup>	2 (10) <sup>b</sup>
		P1- proliferation	12 (60) <sup>a</sup>	14 (70) <sup>a</sup>	1 (5) <sup>b</sup>
Eland (1)	2	Proliferation	2 (100)	2 (100)	2 (100)
		P1-proliferation	2 (100)	2 (100)	0

<sup>1</sup> Total number of replicates (ejaculates).

<sup>abc</sup> Rows with different superscripts are significantly different (P<0.01).

\* Epithelial cell attachment and division could not be distinguished from feeder layer cells.

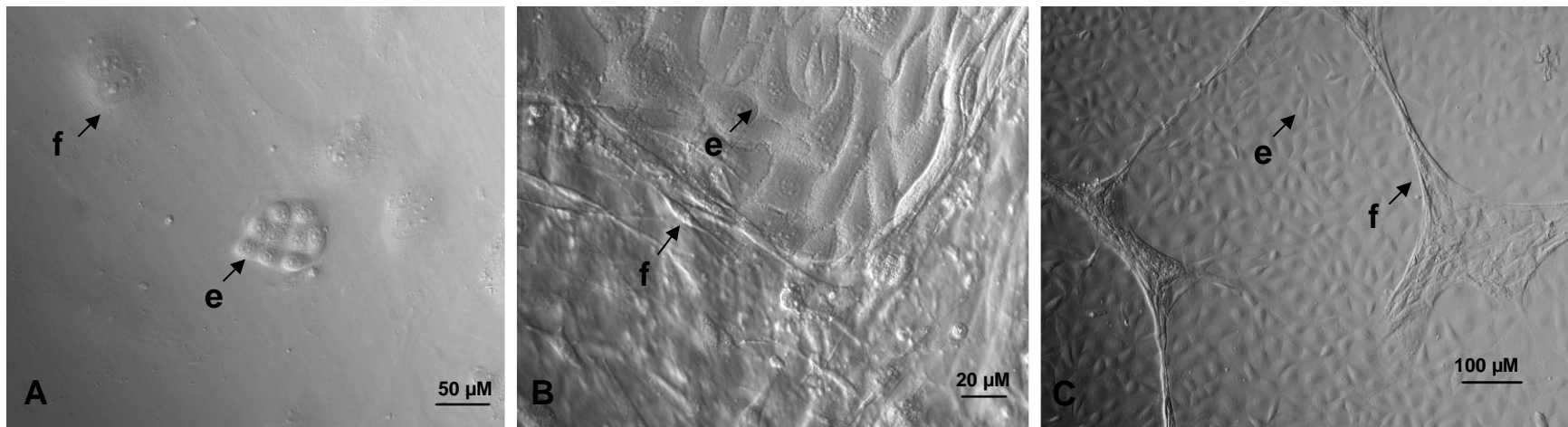


Figure 6.6. Epithelial (e) and fibroblast (f) cells observed in different treatments: Small dividing colony of ram semen-derived epithelial cells on 3T3 feeder layer (A), large, proliferating colony pushing fibroblast cells aside as it expands (B) and confluent P1 cell line with fibroblasts pushed into thin strands(C) (all Hoffman modulation microscopy).

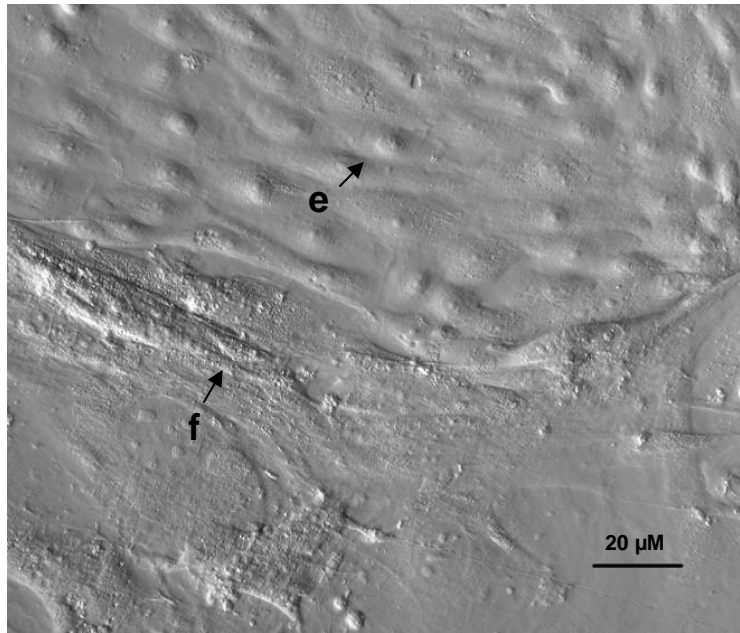


Figure 6.7. Eland semen-derived epithelial-like colony (e) plated on 3T3 feeder cells (f) (Hoffman modulation microscopy).

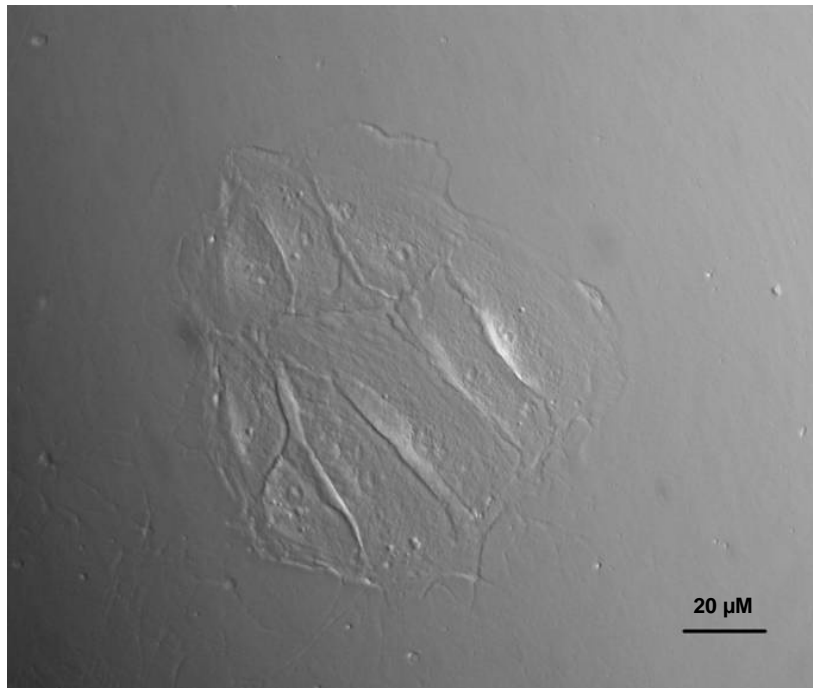


Figure 6.8. Colony of ram epithelial-like colony that ceased proliferation and became senescence (Hoffman modulation microscopy).



Table 6.4. Cell isolation from 20% Percoll fractions of frozen ram and eland semen cultured with feeder cell inserts on collagen.

Species (no. animals)	n <sup>a</sup>	Extender protein	Attached <sup>b</sup>	Proliferate <sup>c</sup>	Epithelial morphology <sup>d</sup>	Microsatellite origin <sup>e</sup>
Ram (4)	4	Egg Yolk	4	4	1	Sheep
Eland (1)	4	Cow Milk or Egg Yolk	4	2	1	Cow*

<sup>a</sup> Number of ejaculates from which five semen straws were processed.

<sup>b</sup> Number of ejaculates from which cell attachment was observed in culture.

<sup>c</sup> Number of ejaculates from which proliferation was observed in culture.

<sup>d</sup> Number of ejaculates from which obtained cells had epithelial morphology. All fibroblast-like cell lines tested positive with mouse markers and were 3T3 fibroblasts.

<sup>e</sup> Species of origin of the semen-derived epithelial-like cells as indicated by DNA microsatellite analysis.

\* Microsatellites amplified with bovine markers, but did not match the eland semen donor, while no amplification was obtained using mouse markers. Thus, the cells were from the milk extender.

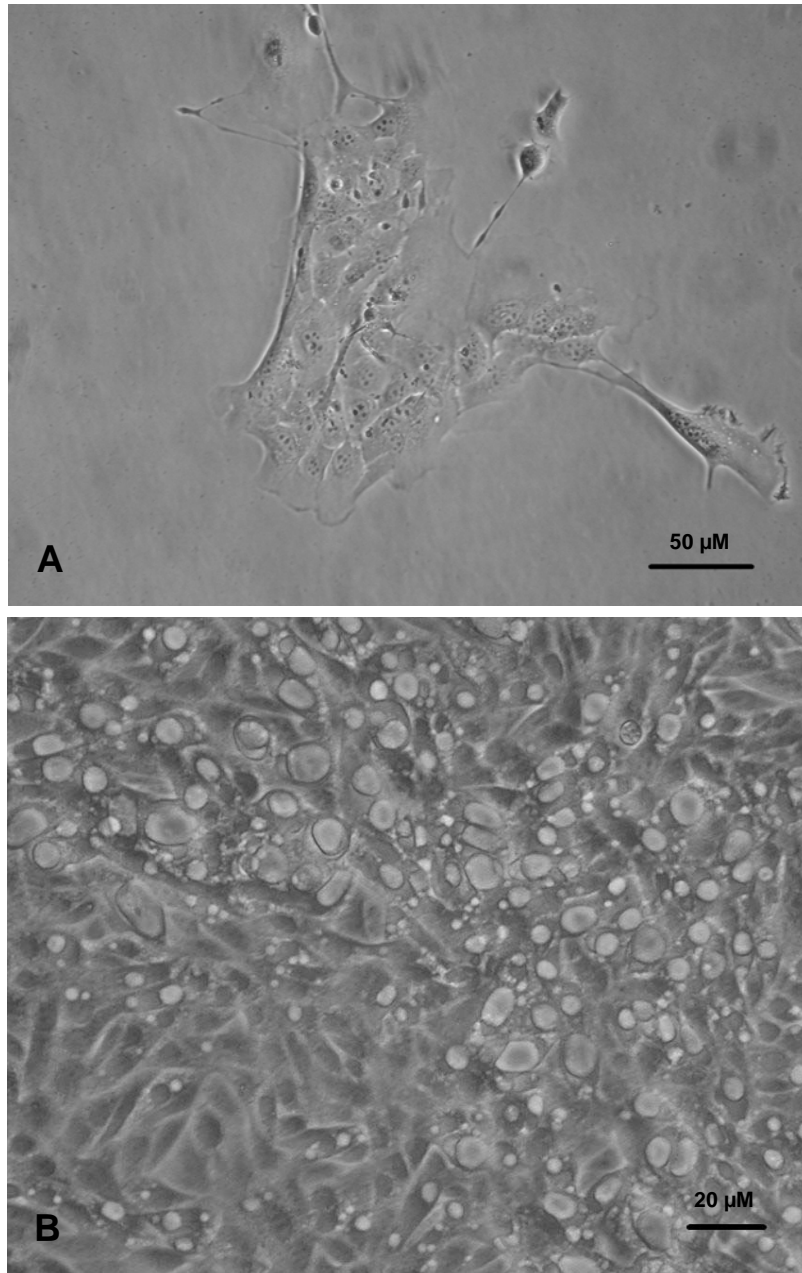


Figure 6.9. Epithelial-like cells isolated and cultured from cryopreserved ram (A) and eland (B) semen.

(effective for testing eland), but did not match the eland bull genotype. Furthermore, the cells formed alveoli-like structures when confluence was reached in culture (Figure 6.9 B).

▪ **Cell characterization by immunocytochemical protein detection and morphology**

Cells that contained keratin fluoresced red (Alexa Fluor® 594), those that contained vimentin fluoresced green (FITC), and positive controls fluoresced red (TRITC). Negative controls did not fluoresce or had very faint auto-fluorescence. All cell nuclei were counterstained with Hoechst 33342 and fluoresced blue. Immunological labeling results and morphology assessment of isolated ram semen cell types are given in Table 6.5. All ram cells co-cultured on 3T3 feeder cells, with inserts or on collagen, expressed keratin, indicating that these cells were of epithelial type.

Keratin was expressed highly by colonies of epithelial-like cells that formed a continuous monolayer with close contact between adjacent cells (Figure 6.10 A). Similarly, colonies that formed a more loosely packed, discontinuous monolayer expressed high levels of keratin (Figure 6.10 B). In contrast, expression of vimentin was low and only in small patches for continuous colonies (Figure 6.10 C), whereas, high vimentin expression was observed in discontinuous colonies with small spaces between adjacent cells (Figure 6.10 D). Vimentin expression for these colonies was localized mainly around the edges of the cytoplasm (Figures 6.10 D and 6.11).

Vimentin and keratin were both detected in 3T3 mouse fibroblast feeder cells (Figure 6.12 A). Therefore, keratin expression could not be used as an exclusive indicator for epithelial cells. The amount of vimentin expression of 3T3 mouse cells was much greater than the expression detected in continuous and discontinuous epithelial-like colonies of ram cells, and was distributed throughout the cytoplasm, while thicker and more defined microtubules were observed (Figure 6.12 B). This expression pattern differed from those observed in epithelial-like cells and therefore, vimentin expression as well as morphology were used as criteria for cell type.

Table 6.5. Cell characterization of somatic cells isolated from ram semen and cultured on feeder cells, with inserts or on collagen, by immunohistochemical detection and morphology.

Treatment	n <sup>a</sup>	Method of cell type assessment				
		Immunohistochemistry <sup>b</sup>		Morphology <sup>c</sup>		
		Keratin	Vimentin	Epithelial	Fibroblastic	Epi + Fibro
Feeder cells	8	8 (100)	4 (50)	5 (63)	2 (25)	1 (13)
Insert	14	14 (100)	10 (71)	8 (57)	5 (36)	1 (7)
Collagen	1	1 (100)	0	1 (100)	0	0

<sup>a</sup> Number of cell lines evaluated.

<sup>b</sup> Number of cell lines that expressed vimentin or keratin. Some cultures expressed both types of cytoplasmic proteins and results indicate number of replicates in which positive staining occurred of the total number of replicates, regardless of overlapping.

<sup>c</sup> Number of replicates which, based on visual microscopic assessment, were of epithelial, fibroblastic or mixed morphology.

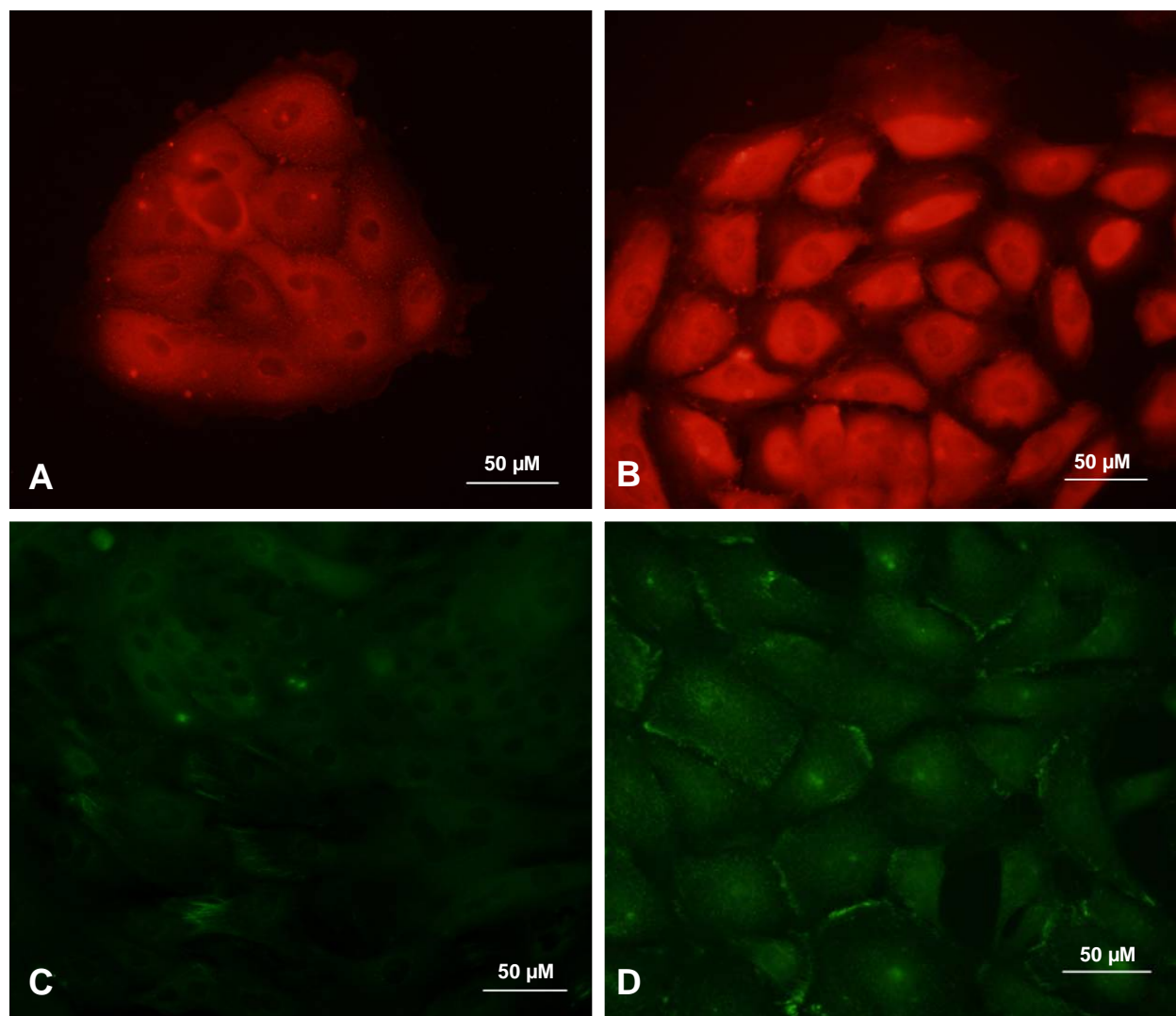


Figure 6.10. Epithelial-like colony of semen-derived ram cells cultured on 3T3 feeder cells expressing keratin (A + B) and vimentin (C + D) under fluorescence microscopy.

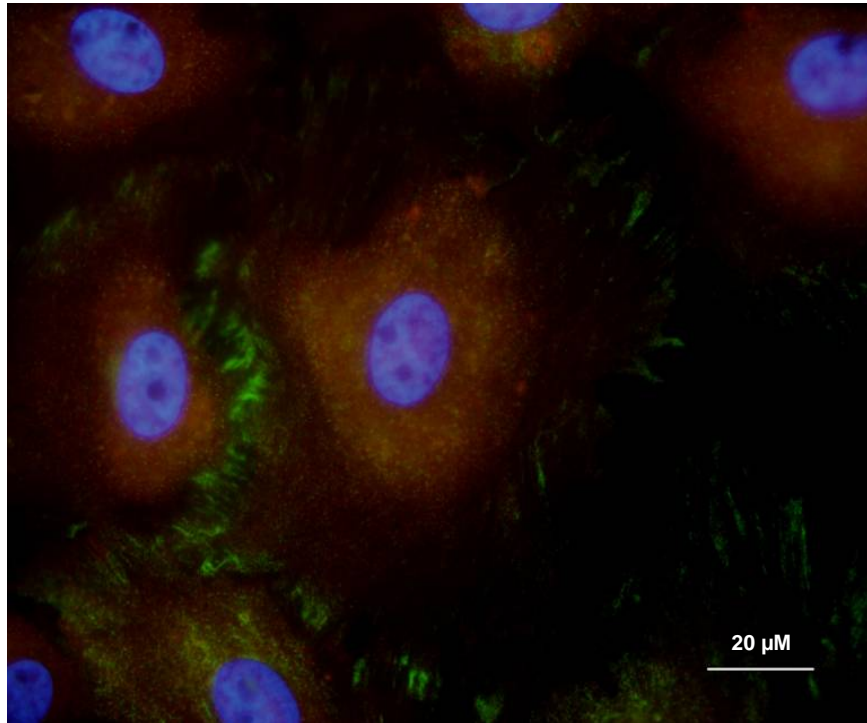


Figure 6.11. Immunological labeling of discontinuous, epithelial-like cells from ram semen with AF594-conjugated anti-keratin (red) and FITC-conjugated anti-vimentin (green) antibodies and counterstained with Hoechst 33342, viewed under fluorescence microscopy.

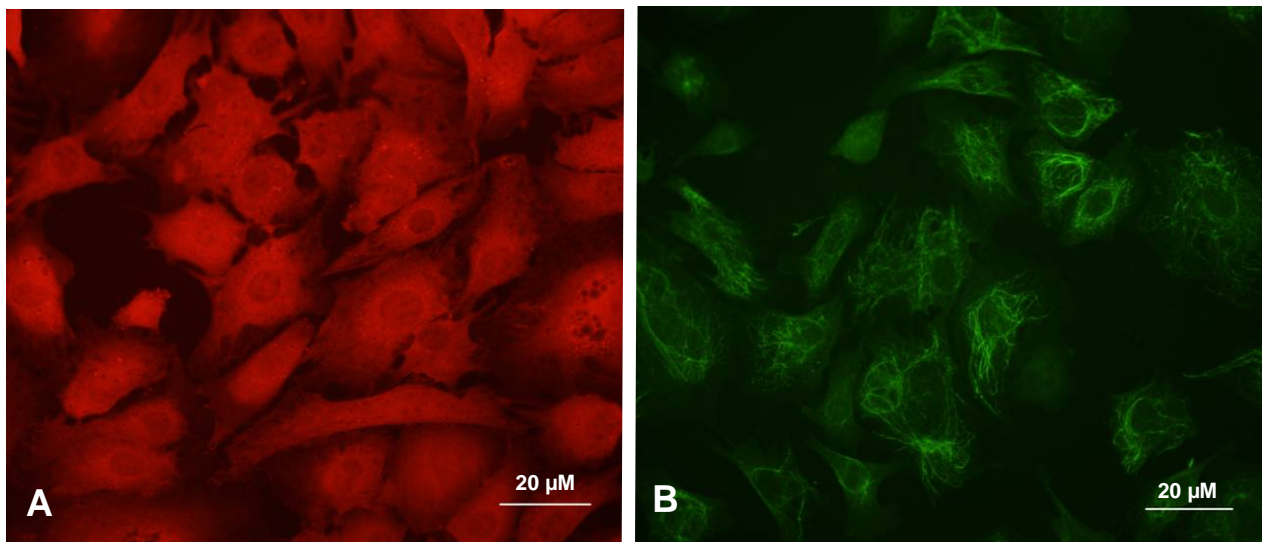


Figure 6.12. Inactivated 3T3 mouse fibroblast feeder cells expressing keratin (A) and vimentin (B) under fluorescence microscopy.

Based on morphology, 88% and 93% of cells isolated and cultured on feeder and insert groups, respectively, contained epithelial-like cells, whereas, 38% and 43% contained fibroblast-like cells. Only one cell line cultured on collagen reached confluence and stained positive for expression of keratin, negative for expression of vimentin and had epithelial morphology (Table 6.5).

## 6.4 Discussion

In the present study, a system that effectively separated epithelial cells from ram and eland semen by Percoll gradient centrifugation was established. Viable epithelial cells were found in the P-20 fraction and *in vitro* cell proliferation was enhanced by co-culture with 3T3 mouse embryonic fibroblasts. Using these procedures, somatic cells were also cultured from cryopreserved semen.

Successful Percoll gradient cell separation from mixed cell type suspensions using a single centrifugation has been demonstrated for blood (Giddings et al., 1980; Segal et al., 1980; Riding and Willadsen, 1981), bone marrow (Olofsson et al., 1980), thymus (Salisbury et al., 1979; Goust and Perry, 1981) and liver (Smedsrod and Pertoft, 1985). Similarly, we were able, with a single centrifugation and different gradient columns, to collect a population of mixed cells that settled at different densities and differed in size and morphology.

Motile and nonmotile spermatozoa were successfully separated and settled in the pellet and P-50 fraction, respectively, following centrifugation with the three-layer Percoll density gradient, as previously shown with Percoll (Sukcharoen, 1994; Yao et al., 1996; Makler et al., 1998; Chen and Bongso, 1999; Palomo et al., 1999; Dode et al., 2002; Suzuki et al., 2003; Walters et al., 2004) and other density gradient micro-beads, such as Ficoll (Palomo et al., 1999), PureSperm (Chen et al., 1999; Allamaneni et al., 2005) or Isolate (Allamaneni et al., 2005). Cell separation is determined by motility and density, as well as cell size (Schumacher et al., 1978) and morphology (Yao et al., 1996). These two factors may have contributed to the



differences in morphology between somatic cells harvested from the different Percoll layers (Figure 6.4), as well as spermatozoa harvested in the pellet and P-50 fractions (Figure 6.5).

The origin, within the reproductive system, of somatic cells isolated from ram and eland semen is unclear, but most likely such cells are of mixed origin, type and age. The rationale of this suggestion is based on the morphological and functional differences among areas or zones along the tract. For example, the six zones of the epididymis each possess cellular differences (Clermont and Flannery, 1970) and within each zone a mixture of cell types were found, including basal, secretory epithelial, endocrine, stem cells and leucocytes (Clermont et al., 1970; Dym and Romrell, 1975; Oatley et al., 2004; Peehl, 2005).

Phillips et al. (1978) used human semen as a source of epithelial cells for *in vitro* culture, although their origin was not described. These cells were polygonal with typical epithelial characteristics and were not contaminated by fibroblast. Others have collected epithelial cells from different portions of the male reproductive tract, including Leydig and Sertoli cells (Welch et al., 1979; Nakhla et al., 1984; Renier et al., 1987; Bilinska, 1989), the ductuli efferentes (Byers et al., 1985b; Raczek et al., 1994; Chen et al., 1998; Janssen et al., 1998), different zones of the epididymis (Clermont et al., 1970; Kierszenbaum et al., 1981; Klinefelter et al., 1982; Wagley et al., 1984; Byers et al., 1985a; Joshi, 1985; Paulson et al., 1985; Finaz et al., 1991; Raczek et al., 1994; Chen et al., 1998; Bassols et al., 2004), prostate (Stonington and Hemmingsen, 1971; Webber et al., 1974; Rose et al., 1975; Burleigh et al., 1980; Eaton and Pierrepont, 1982; Merk et al., 1984), seminal vesicles (Phillips and Rice, 1983; Mata et al., 1986; Tsuji et al., 1994; Shima et al., 1995), bulbourethral glands (Shima et al., 1995) and urethra (Petzoldt et al., 1994; Harvey et al., 1997).

Morphological descriptions and photomicrographs presented in mentioned reports correspond to the characteristics of epithelial cells observed in our study. Epithelial cells collected from different replicates did not always appear to be of the same morphological type and occasionally cells of two different epithelial morphologies were present in a single culture,

which contradicts finding by Phillips et al. (1978) where only one type of epithelial cell was observed. Accordingly, our findings indicate that the specific origin of the cell population in semen is variable. Specific epithelial cell types could not be distinguished by the methods we used; therefore, further studies are needed to determine the specific origin and properties of the isolated cells.

Attachment of cells isolated from the different Percoll fractions was consistent with Trypan Blue staining confirming that most viable cells were isolated in the P-20 layer, although cells occasionally plated and formed colonies from the P-0 and P-50 layers. Even though a different cell type was obtained from P-50 fraction that was smaller in size than cells from the other layers, such cells did not attach or grow on the culture surface. Likewise, no cells isolated from the P-90 layer attached or grew in culture. Clearly, the viable cells remained in the upper fractions of the gradient column. Furthermore even if live somatic cells were in the pellet, it is highly unlikely that attachment could have occurred within the first 24 hours of culture due to the currents in the medium caused by millions of progressively motile spermatozoa also present in the pellet and the lack of access to the surface of the dish due to spermatozoa already attached. Also, since the somatic cells are mixed with high concentration of motile spermatozoa, the energy sources will be more rapidly depleted and, in turn, the metabolites could alter the pH of the culture medium.

Although quantification was not done, the isolation of cells by discontinuous gradient centrifugation did not seem to influence cell growth and proliferation after attachment and the initial change of medium. Therefore, the present separation technique enhances the ability of somatic cells to contact the culture surface without physical and biochemical interference by highly motile spermatozoa.

It is well known that most epithelial cells require specific culture conditions for optimum proliferation. Inactivated 3T3 mouse fibroblast cells are frequently used as feeder cells with many types of epithelial cells (Freshney, 2002). Cell proliferation and life span of several

epithelial-type cells such as cells from the epidermis (Rheinwald and Green, 1975), mammary (Taylor-Papadimitriou et al., 1977), trachea (Gray et al., 1983; Lee et al., 1984), thymus (Sun et al., 1984), middle ear (van Blitterswijk et al., 1986) and prostate (Kabalin et al., 1989) have been improved by co-culture with 3T3 cells. Likewise, in our study cell proliferation was enhanced when semen-derived cells were plated directly on 3T3 mouse feeder cells or co-cultured with 3T3 inserts compared with cells plated directly on collagen.

Fibroblasts improve the culture environment by altering the growth surface, as well as secreting beneficial growth factors. Among other proteins, feeder cells are known to produce laminin, fibronectin and Type 4 collagen (Alitalo et al., 1982; Wu et al., 1982), major components of the basal lamina of the extracellular matrix *in vivo* that may promote epithelial attachment to the culture surface. However, some epithelial cells also produce variants of these structural proteins in culture, such as epidermal keratinocytes (Alitalo et al., 1982). This may partially explain why some epithelial cells can be cultured successfully in conditioned medium without surface modifications (Gray et al., 1983; Kabalin et al., 1989). Wu et al. (1982) reported that proliferation of epithelial cells from the trachea required the presence of 3T3 cells, as well as a collagen substratum. This observation suggests that epithelial cells need an altered growth surface as well as proliferation stimulating factors present in medium conditioned by the 3T3 cells.

While conditioning medium before culture may provide the same benefits as co-culture for some cells (Kabalin et al., 1989), other cell types, such as mammary cells, require co-culture with 3T3 cells and it is suggested that the beneficial factors may have low stability in medium and need constant replenishment by fibroblasts (Taylor-Papadimitriou et al., 1977). Such contradictory results indicate that different types of epithelial cells behave differently in culture and have unique requirements for optimal *in vitro* proliferation. We did not observe differences between the two different 3T3 culture systems, suggesting that contact between fibroblast feeders and epithelial cells is not necessary for enhancing cell proliferation of semen-derived

epithelial cells. Coating culture plates with collagen before plating the 3T3 fibroblasts may have promoted epithelial attachment, but further research is required to determine the specific role of collagen coating on cell proliferation.

Directional secretion of growth factors have been described for several cell types, including placental (Hemmings et al., 2001; Wyrwoll et al., 2005), endometrial (Hornung et al., 1998), epididymal (Cooper et al., 1989) and Sertoli cells (Spaliviero and Handelsman, 1991). Wyrwoll et al. (2005) demonstrated that  $^3\text{H}$ -inulin can diffuse freely through 1  $\mu\text{m}$  membrane pores in the absence of cells. However, when a confluent monolayer of human choriocarcinoma cells was present, this diffusion was terminated.

In the present study, proliferation of epithelial cells was similar between cells that were cultured on feeders and those that were separated from feeder cells by a membrane insert. One explanation may be that if directional growth factor secretion by fibroblasts did occur to the apical side only, the seeding density was low enough to leave some membrane surface uncovered and permit diffusion of the factors through the membrane pores to the basal medium compartment where epithelial cells were cultured. It is also possible that the 3T3 fibroblasts secrete growth factors bidirectionally and therefore, these factors were likely released into the chamber beneath the membrane through the pores. Further research on directional secretion of growth factors by 3T3 cells may shed some light on this subject.

Immunohistochemical detection of cytoskeletal proteins revealed that all somatic cells from semen contained keratin, a positive indication of epithelial origin. However, 3T3 mouse fibroblasts also labeled positive for keratin and the supplier confirmed that these feeder cells dramatically cross react with keratin antibodies. Therefore, keratin labeling could not be used to distinguish between feeder and semen-derived cells in culture. Inactivation of feeder cells is not 100% and there is a possibility that some fibroblasts may have proliferated in culture. Although highly unlikely, there is also a possibility that some fibroblasts migrated through the insert membrane and plated on the collagen-coated surface. It is, therefore, essential to be able to

distinguish feeder cells from epithelial cells. Morphology and vimentin expression were examined to identify epithelial-like cells in semen. Fibroblast-like cells that stained negative for keratin were not observed, indicating that fibroblast-like cells were probably 3T3 cells and not fibroblasts from the semen donor.

Vimentin is known to play a role in epithelial cell migration (Gilles et al., 1999), and this may explain the expression of vimentin in the more dissociated epithelial-like colonies. It is also possible that different populations of cells came from different regions of the male reproductive tract, or that other variables such as age of founding colonies, caused the behavioral differences during culture. Epithelial cells are prone to change morphology, especially during suboptimal *in vitro* conditions. Epithelial-mesenchymal transition confers resistance to the apoptotic effects of Transforming Growth Factor-Beta in hepatocytes (and are characterized by more fibroblastic shape and a replacement of cytokeratin with vimentin in epithelial cells (Valdes et al., 2002). It is therefore possible that the vimentin expression observed in epithelial cells in the present study was acquired during culture.

Following results from the Percoll isolation and 3T3 co-culture experiments, these methods were used to successfully obtain proliferating cell lines from cryopreserved ram and eland semen, which was unsuccessful until now. Clearly, the improved system was beneficial for somatic cells from cryopreserved semen as compared with the unimproved system previously used. DNA microsatellite analysis confirmed that one of the five cell lines from cryopreserved ram semen, the only one with epithelial morphology, was indeed ram cells. The DNA results confirmed that the fibroblast-like cells from both ram and eland semen were 3T3 cells. These results suggest that feeder cell contamination can occur through the 1  $\mu$ m membrane pores, since extra precautions were taken not to contaminate the semen-derived cells with 3T3 cells during medium changes. Therefore, it is important to establish a system where co-culture can be eliminated, such as the use of conditioned or defined medium.

One epithelial-like colony was also derived from cryopreserved eland semen. Surprisingly, the microsatellite analysis indicated that the epithelial cells obtained from frozen-thawed eland semen were not from the eland semen donor, but amplified with bovine markers and not murine markers. Bovine milk was used as a cryopreservation medium for eland semen. However, before milk is used as an extender it is heated to 95°C for 10 minutes to inactivate the toxic factors of its protein fraction (Evans and Salamon, 1987). It is remarkable that somatic cells survived such high temperatures and remained mitotically active. Another indication that these cells originated from the milk was alveoli-like structures that formed in the epithelial-like colonies, which is characteristic of mammary epithelial cells (Rose et al., 2002). Therefore, we are confident that the epithelial cells originated in the cow mammary gland and survived heating as well as the semen cryopreservation process. These findings should be considered for future semen freezing protocols.

In conclusion we have established a system that effectively separated and supported *in vitro* proliferation of epithelial cells from semen. The technique may be useful for obtaining cells for otherwise impossible propagation of offspring by nuclear transfer of genetically valuable domestic and endangered animals. The culture system may be simplified by further research on growth requirements. Also, the elimination of feeder fibroblasts would improve practical application and prevent contamination of semen-derived cell lines.

## CHAPTER 7

### INTERGENERIC NUCLEAR TRANSFER OF SEMEN-DERIVED ELAND EPITHELIAL CELLS INTO BOVINE OOCYTES

#### 7.1 Introduction

Since the birth of the first lamb cloned from an adult somatic cell (Wilmut et al., 1997), cloning technology has been used not only in basic research, but also for the multiplication of elite livestock, production of transgenic animals and conservation of endangered species (Campbell et al., 2005). Nuclear transfer (NT) could be especially valuable in conservation of species that are endangered due to inbreeding, because introduction of novel genes would dilute the effects of deleterious recessive genes (Ryder et al., 1997).

Fresh or cryopreserved donor cells can be used for NT procedures. Somatic cells of various types, such as cumulus (Kato et al., 1998, 2000; Wayakama et al., 1998), mammary epithelial (Wilmut et al., 1997; Kishi et al., 2000), oviduct (Kato et al., 1998, 2000), fibroblast cells (Baguisi et al., 1999) and leucocytes (Galli et al., 1999) have been used to create cloned animals. Until now, if cell lines were not derived and cryopreserved from either the live or recently deceased animal, no nuclear donor material was available. The possibility of using somatic cells isolated from cryopreserved semen for NT would enable the re-activation of previously unavailable genes from earlier generations. Therefore, banks of cryopreserved semen are a potential source of novel genes that may soon be able to be re-incorporated into the gene pool. Recently, it was demonstrated that epithelial cells from sheep (*Ovis aries*) and Common eland antelope (*Taurotragus oryx*) semen can be successfully isolated and used to establish cell lines *in vitro* (Nel-Themaat et al., 2004, 2005).

Cloned embryos are usually derived by the transfer of an adult or fetal somatic cell into a conspecific oocyte (intraspecies) (Wilmut et al., 1997; Kato et al., 1998; Wayakama et al., 1998; Polejaeva et al., 2000; Galli et al., 2003). However, since oocytes from endangered

species are rarely available, oocytes of closely related domestic or laboratory animals are used as recipient cytoplasts. Pregnancies in the banteng (Sansinena et al., 2005) and live births of cloned endangered mammals, such as gaur (Lanza et al., 2000), mouflon (Loi et al., 2001), banteng (Holden, 2003) and African wildcats (Gómez et al., 2004) have been produced by intrageneric NT.

In contrast, intergeneric nuclear transfer (igNT) has been less successful and viable mammalian progeny have not been produced. Even though no live mammals have been produced by igNT, early embryo development has been achieved after igNT of saola (Bui et al., 2002), bongo (Lee et al., 2003), bison (Lu et al., 2005), African buffalo, bontebok, eland (Matshikiza et al., 2004), yak and dog (Murakami et al., 2005) somatic cells into bovine oocytes with cleavage rates similar to that of bovine NT embryos. Development was arrested in a high frequency of igNT cloned embryos and was coincident with embryonic genome activation (Lee et al., 2003; Matshikiza et al., 2004; Lu et al., 2005; Murakami et al., 2005). Thus, few igNT embryos developed to the blastocyst stage (Bui et al., 2002; Lee et al., 2003; Ty et al., 2003; Matshikiza et al., 2004; Lu et al., 2005; Murakami et al., 2005). The high incidence of developmental arrest of igNT embryos are likely due to multiple factors, but arrest at the time of embryonic genome activation may be associated with abnormal nuclear remodeling (Hamilton et al., 2004).

The oocyte cytoplasm controls nuclear events and DNA replication of the transplanted nuclei (Campbell et al., 1993). Therefore, cytoplasmic incompatibilities between the nucleus and somatic cell may contribute to the abnormal nuclear remodeling.

In the present study, we evaluated the ability of eland epithelial cells isolated from semen to support early *in vitro* embryonic development when transplanted to bovine cytoplasts. Also, we examined the ability of eland cells to initiate DNA replication after transplantation as compared to that of bovine NT embryos.



## **7.2 Materials and Methods**

### **▪ Experimental design**

In the first experiment, the three treatments of embryo production were: (1) NT using bovine fibroblasts, (2) igNT using semen-derived eland epithelial cells and (3) parthenogenetically activated oocytes. The number of embryos that (a) cleaved, (b) developed to the morula stage and (c) developed to the blastocyst stage were assessed, while the blastocyst cell numbers were compared across treatments.

In the next experiment, treatments were determining DNA synthesis (1) before activation, (2) 16 hours after activation and (3) 72 hours after activation between bovine NT, eland igNT and parthenogenetic embryos. For the 16 hours after activation treatment, nuclear stages were compared among bovine NT, eland igNT and parthenogenetic embryos. Stages assessed were (a) swollen nucleus, (b) premature chromatin condensation and (c) 2-cell. For the 72 hours after activation treatment, embryos were divided into two groups (2 to 7 cells and  $\geq 8$  cells) and DNA synthesis was compared between bovine NT and eland igNT embryos within and between bovine NT and eland igNT embryo groups.

### **▪ Animal**

A 6-year old Common eland bull with a body weight of ~530 kg was maintained on a diet consisting of 8 kg of herbivore feed (Purina ADF-16) at the Freeport-McMoRan Audubon Species Survival Center (FMA-SSC) near New Orleans, Louisiana. The Institutional Animal Care and Use Committee of FMA-SSC approved all eland procedures.

### **▪ Chemical reagents**

All chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA), unless otherwise stated.

#### ▪ Establishment of donor cell line

An eland epithelial cell line was established as described previously (Nel-Themaat et al., 2005). Briefly, eland semen was collected by a combination of rectal massage and electroejaculation (Wirtu et al., 2002b) and washed by diluting in 10 mL of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free Dulbecco's phosphate-buffered saline (DPBS, Gibco, Grand Island, NY, USA) and centrifuging at 500 x g for 10 minutes. Samples were washed two more times and the final pellet was resuspended in 2.5 mL of culture medium (CM) consisting of minimum essential medium alpha medium ( $\alpha$ -MEM; Gibco) supplemented with 15% newborn calf serum (NCS), 1% MEM nonessential amino acids (NEAAs), 500 IU/mL of penicillin and 0.5 mg/mL of streptomycin (5% Pen/Strep; Cellgro, Herndon, VA, USA) and 250  $\mu\text{g/mL}$  of gentamicin (Gibco). The resuspended pellet was plated on collagen-coated (Type 1 from calf skin) 35-mm culture plates and cultured for 24 hours before rinsing with CM containing 1% Pen/Strep and no gentamicin. *In vitro* culture continued until cell colonies covered approximately 0.5  $\text{cm}^2$  of the culture surface. Colonies were dissociated for subpassages using 0.125% trypsin in DPBS and cultured until passages 3 to 5 before cryopreservation in CM plus 10% DMSO. In the evening before each NT session, a vial of frozen eland epithelial cells was thawed and plated on a collagen-coated culture plate. Immediately before NT, cells were dissociated (as described above) and resuspended in CM.

The bovine fibroblast cell line was obtained by mincing an ear biopsy of a 4-year old crossbred beef type domestic cow and culturing the minced tissue in a 35-mm culture plate in CM until confluent. Cells were dissociated using 0.25% trypsin in DPBS and resuspended in 25- $\text{cm}^2$  flasks. At passages 3 to 5, confluent cells were dissociated and cryopreserved in CM plus 10% DMSO in aliquots for NT. In the evening before bovine NT sessions, one vial of bovine cells were thawed and suspended in CM in a 35- $\text{mm}^2$  culture dish. Similarly to eland epithelial cells, bovine fibroblast cells were dissociated immediately before the NT procedure.

#### ▪ Analysis of cell-cycle phase by flow cytometry

Eland epithelial cells (passages 3 to 5) were dissociated from the culture flasks, centrifuged at 300 x g for 5 minutes and diluted to approximately  $1 \times 10^6$  cells/mL. Duplicate samples were stained with 50 µg/mL of propidium iodide (PI), 650,000 units/mL of RNase and 0.1% (v/v) Triton X-100 (Crissman and Steinkamp, 1973) for 20 minutes at 24°C.

Data from ~10,000 nuclei per sample were acquired in doublet discrimination mode using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) flow cytometer equipped with an argon laser. Propidium iodide is excited at 488 nm and after binding with DNA, emits red fluorescence at 510 nm. Data were collected using CELLQuest software (Becton Dickinson) with FL2-area and a linear scale of fluorescence. One parameter (FL2-A) 1024-channel histograms and two parameter (FL-2 A and FL-2 W) 1024-channel dot plots were generated and analyzed with Modfit LT cell cycle analysis software (Verity Software House, Inc., Topsham, ME) using F\_DIP\_T1.mod.

#### ▪ Oocyte maturation

Cow ovaries were obtained from a local slaughterhouse and transported to the laboratory at 30°C in saline solution (0.9%) containing 1% Pen/Strep. Cumulus-oocyte complexes (COCs) were aspirated from follicles (3 to 10 mm) and cultured in modified TCM-199 containing 1 IU/mL of human chorionic gonadotropin (hCG), 0.5 IU/mL of equine chorionic gonadotropin (eCG), 10 µg/mL of epidermal growth factor (EGF) and 3 mg/mL of bovine serum albumin (BSA, fraction V, fatty acid free; Serological Proteins, Kankakee, IL, USA) for 19 hours in 5% CO<sub>2</sub> in air at 38.5°C.

Cumulus cells of *in vitro*-matured oocytes were removed by vortexing in 1mg/mL of hyaluronidase for 3 minutes. Denuded oocytes were placed in CR1aa medium (Rosenkrans and First, 1994) supplemented with 5% NCS, 1% NEAA, 2% Basal Medium Eagle essential amino acids (EAA, Gibco), 1 mM glutamine and 50 µg/mL of gentamicin at 38.5°C in 5% CO<sub>2</sub> in air until further use.

## ▪ Nuclear transfer

To eliminate chances of cross-contamination between eland and bovine cells and to reduce variability, NT for each species was executed on alternating days using oocytes from the same source.

Before enucleation, denuded metaphase II (M-II) oocytes were incubated for 10 to 30 minutes in CR1aa medium containing 10 µg/mL of Hoechst 33342 and 7.5 µg/mL of cytochalasin B (CCB). After incubation, oocytes were enucleated in HEPES-buffered TCM-199 supplemented with 15 mM HEPES, 15 mM NaHCO<sub>3</sub>, 0.36 mM pyruvate, 1 mM glutamine, 2.2 mM calcium lactate, 50 µg/mL of gentamicin, 0.4% BSA and 10 µg/mL of CCB. The M-II plate was aspirated into an enucleation pipette (outer diameter 20 to 25 µm) and where possible, the first polar body was removed. Removal of the metaphase spindle was confirmed by brief exposure (~5 seconds) to ultraviolet (UV) light using epifluorescence microscopy.

A single eland epithelial or bovine fibroblast cell was introduced into the perivitelline space of each enucleated oocyte. For fusion, each couplet was placed in a solution of 0.3 M mannitol and 0.1 mM Mg<sup>2+</sup> between two stainless steel electrodes spaced 120 µm apart (LF-101; Nepa Gene, Tokyo, Japan). Membrane fusion was induced by applying a 0.2-second AC prepulse of 17 V, 1 MHz; followed by two 35-µsecond. DC pulses of 14 V at intervals of 1 second. Following the fusion pulses, couplets were cultured in CR1aa and after 20 to 30 minutes, fusion was assessed visually by confirming the presence or absence of the donor cell in the perivitelline space. Fused couplets were cultured for 2 to 3 hours in CR1aa medium supplemented with 7.8 mM calcium lactate and 2.5 µg/mL of CCB. Activation of fused couplets was performed by incubation in 10 µM calcium ionophore for 5 minutes and subsequent culture for 4 hours in 2 mM dimethylaminopurine (DMAP) at 38°C in 5% CO<sub>2</sub> in air under mineral oil. As a control for the activation protocol, some denuded M-II oocytes were parthenogenetically activated by the same procedure as described for the fused NT couplets.

Following activation, NT couplets and parthenogenetically activated oocytes (PAOs) were cultured in CR1aa medium under mineral oil in 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> at 38.5°C for 9 days.

#### ▪ Embryo evaluation

Fused couplets and PAOs were evaluated for frequency of cleavage on day 3 and development to the blastocyst stage on days 7, 8 and 9. All evaluations were done visually using stereomicroscopy. Blastocyst cell numbers, both for the inner cellular mass (ICM) and trophodectoderm (TPD), were determined using a modification of a previously described protocol (Wells, 2000).

Couplets that did not cleave by day 3 were fixed at 4°C for 24 to 48 hours in ethanol and acetic acid (3:1) and stained with aceto-orcein. Uncleaved couplets were examined using phase-contrast microscopy (400X) for nuclear status.

Cleaved NT embryos that had fewer than four cells on day 3, as well as parthenogenetically activated and NT embryos that did not develop to the blastocyst stage by day 9, were incubated for 30 to 60 minutes at 38°C in 20 µg/mL of Hoechst 33342. Stained embryos were placed in a drop of antifade reagent (FluoroGuard™) on a clean glass microslide and cell numbers were counted by epifluorescence microscopy.

Nuclear transfer and parthenogenetic blastocysts on days 7, 8 and 9 were cultured for 1 hour in Hoechst 33342 as described above. After incubation, blastocysts were exposed to 0.04% Triton X-100 in PBS for 30 seconds and then cultured for 15 minutes at 38°C in 25 µg/mL of PI. Stained blastocysts were then mounted on microscope slides as described above and the ratio of ICM cells to TPD cells per embryo was determined by counting the number of blue (ICM) and red (TPD) cells stained by Hoechst and PI, respectively.

#### ▪ Determination of DNA synthesis

Replication potential of eland epithelial or bovine fibroblast cells transferred into *in vitro* matured bovine oocytes was evaluated by immunocytochemical detection of 5-bromo-2' -

deoxyuridine 5'-triphosphate (BrdU; Roche Diagnostics Corporation, Indianapolis, IN, USA) incorporated into newly synthesized DNA strands.

To detect DNA replication, eland and bovine NT cybrids (1-cell embryos that contain cytoplasm from the oocyte and the somatic cell) or embryos were incubated in BrdU at different times after NT: (1) nonactivated cybrids (2 to 3 hours after fusion), (2) activated cybrids (4 hpa, fixed at 16 hpa), (3) cleaved NT embryos (72 hpa, fixed at 84 hpa) and (4) bovine PAOs (4 or 72 hpa, fixed at 16 and 84 hpa, respectively).

Oocytes, cybrids and embryos (OCEs) in each treatment were incubated in 20  $\mu$ M BrdU at 38°C for 2 hours (Treatment 1) or 12 hours (Treatments 2, 3 and 4), then washed three times in PBS plus 5% NCS (PBS-S). After labeling, the zona pellucida was removed by incubation in 0.3% pronase for 3 to 5 minutes and rinsed in PBS-S. Zona-free OCEs were immediately fixed in a solution of 2.5% paraformaldehyde for 24 hours at 4°C. Fixed OCEs were rinsed in PBS-S and membranes were permeabilized in PBS supplemented with 0.1% of Triton X-100 for 10 minutes. After permeabilization, OCEs were rinsed in PBS supplemented with 0.1% Tween 20 (PBS-T20) and finally cultured in 7.5 mg/mL of glycine in PBS-T20 for 10 minutes to prevent nonspecific binding. Then, OCEs were rinsed in PBS-T20 and DNA denaturation was performed by 30 minutes of incubation in 2N HCl. Oocytes, cybrids and embryos were incubated in PBS-S for 5 minutes and then in incubation buffer consisting of PBS-T20 plus 0.5% BSA for 10 minutes, before exposure to 50  $\mu$ L of monoclonal anti-BrdU antibody conjugated with FITC for 45 minutes at 38°C. Finally, chromatin was stained with 25  $\mu$ g/mL of PI in PBS-S for 5 minutes and OCEs were rinsed twice for 3 and 15 minutes before being mounted on clean glass microscope slides and covered with mounting medium before viewing with fluorescence microscopy as described above.

When DNA synthesis was evaluated by epifluorescence microscopy, nuclei that incorporated BrdU (S-phase) fluoresced green, whereas, the total number of nuclei fluoresced red. Nuclear stages of embryos were also assessed and total cell numbers were counted.

### ▪ **Statistical analysis**

Results were analyzed using Graphpad Instate software (Graphpad Software, Inc., San Diego, CA, USA) and a P-value of less than 0.05 was considered significant for all experiments. Developmental comparisons between bovine NT and eland igNT embryos were performed using the Chi-square analysis, while percentage of blastomeres that contributed to the ICM and TPD were compared by Unpaired t-tests with a Welch correction. The numbers of cells that incorporated BrdU between cell number groups (2 to 7 cells or  $\geq 8$  cells) were compared using Fisher's Exact test.

## **7.3 Results**

### ▪ **Analysis of cell cycle by flow cytometry**

Flow cytometry analyses revealed that most eland epithelial cells were in the G<sub>0</sub>/G<sub>1</sub> phase (82%) while lower proportions of cells were in the S phase (10%) and G<sub>2</sub>/M (10%) phases (Figure 7.1).

### ▪ **Nuclear transfer**

Nuclear transfer results are presented in Table 7.1. Fusion efficiency and cleavage rates were similar when eland epithelial cells (90.8% and 82.7%, respectively) (Figure 7.2) were used as donor karyoplasts, as compared with bovine fibroblasts (93.7% and 80.0%, respectively). Development to morula and blastocyst stages were similar for bovine NT embryos (38% and 21%, respectively) and PAOs (42.6% and 32.7%, respectively). Development of morulae, however, was lower ( $P < 0.001$ ) for eland igNT embryos (0.5%) and no eland igNT embryos developed to the blastocyst stage.

The mean total cell number in bovine NT blastocysts ( $76 \pm 6.1$ ; Figure 7.3 A) was similar to that of blastocysts from PAOs ( $76 \pm 8.4$ ) (Figure 7.3 B). Although ICM cell numbers in the bovine NT blastocysts (22) and parthenogenetic bovine blastocysts (14) were similar, the

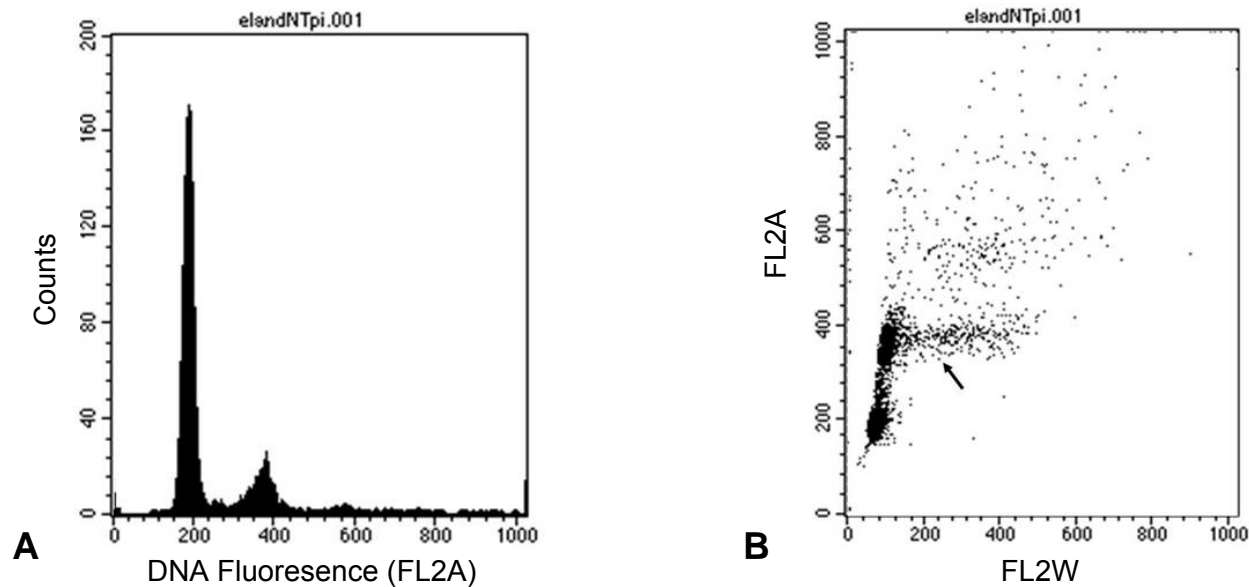


Figure 7.1. Flow cytometric histogram (A) showing the cell cycle of epithelial cells isolated from eland semen. The leftmost peak represents the G<sub>0</sub>/G<sub>1</sub> cells with 2C amount of DNA, the rightmost peak represents cells in G<sub>2</sub>/M phase with 4C amount of DNA, and the cells in between the peaks are in the S phase undergoing DNA synthesis, having amounts of DNA between 2C and 4C. The y-axis represents the nuclei numbers and the x-axis represents the PI fluorescence intensity that translates to the quantity of DNA per cell. Flow cytometric dot plot (B) shows fluorescence due to width and area of the nuclei from epithelial cells isolated from eland sperm. The model used in the software for determining the frequency of events in the cell cycle phases ensures aggregates are not counted, as in the arrow at G<sub>2</sub>/M.



Table 7.1. Development of embryos from nuclear transfer of bovine fibroblasts and eland semen-epithelial cells into bovine oocytes, and parthenogenetically activated bovine oocytes.

Donor cell type	COCs <sup>1</sup>	Fused couplets	Cleaved day 3 n (%) <sup>2</sup>	Morulae day 7 n (%) <sup>3</sup>	Blastocysts days 7 to 9		
					Day 7 to 9 n (%) <sup>3</sup>	TPD cell no. <sup>4</sup> (%)	ICM cell no. <sup>4</sup> (%)
Bovine fibroblasts	128	120	96 (80)	36 (38) <sup>a</sup>	20 (21) <sup>a</sup>	53±5.0 (70±4.0)	22±3.9 (29±4.0)
Eland semen epithelial	230	209	173 (83)	9 (0.5%) <sup>b</sup>	0 <sup>b</sup>	-	-
Parthenogenetic activation	75	N/A	61 (81)	26 (43) <sup>a</sup>	20 (33) <sup>a</sup>	67±6.5 (82±4.5)	15±3.8 (19±4.7)

<sup>1</sup> Number of cumulus-oocyte-complexes that were produced during NT.

<sup>2</sup> Percentage from total fused couplets.

<sup>3</sup> Percentage from total cleaved embryos.

<sup>4</sup> Data presented as mean±SE.

<sup>abc</sup> Different superscripts within columns are significantly different (P<0.001).

Mean ICM % derived as mean of percentage of the total cell number (ICM + TPD) in each embryo.

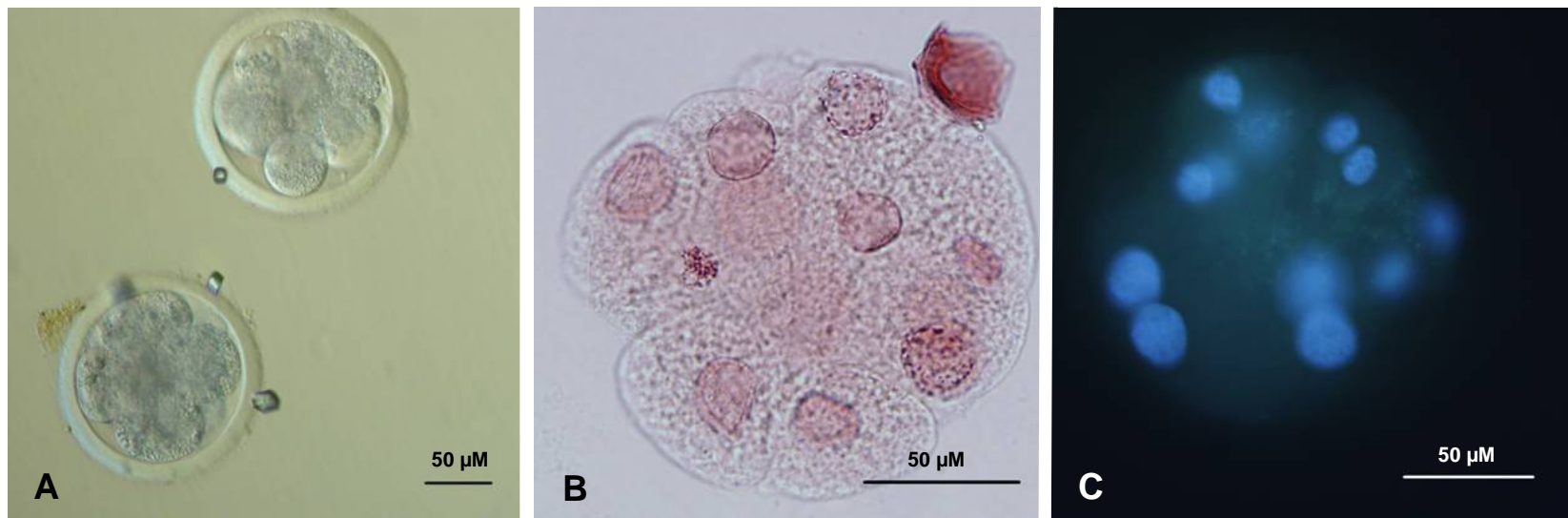


Figure 7.2. Eland day-3 to day-4 igNT embryos (A), stained with aceto-orcein (B) and stained with Hoechst 33342 under epifluoresence microscopy (C).

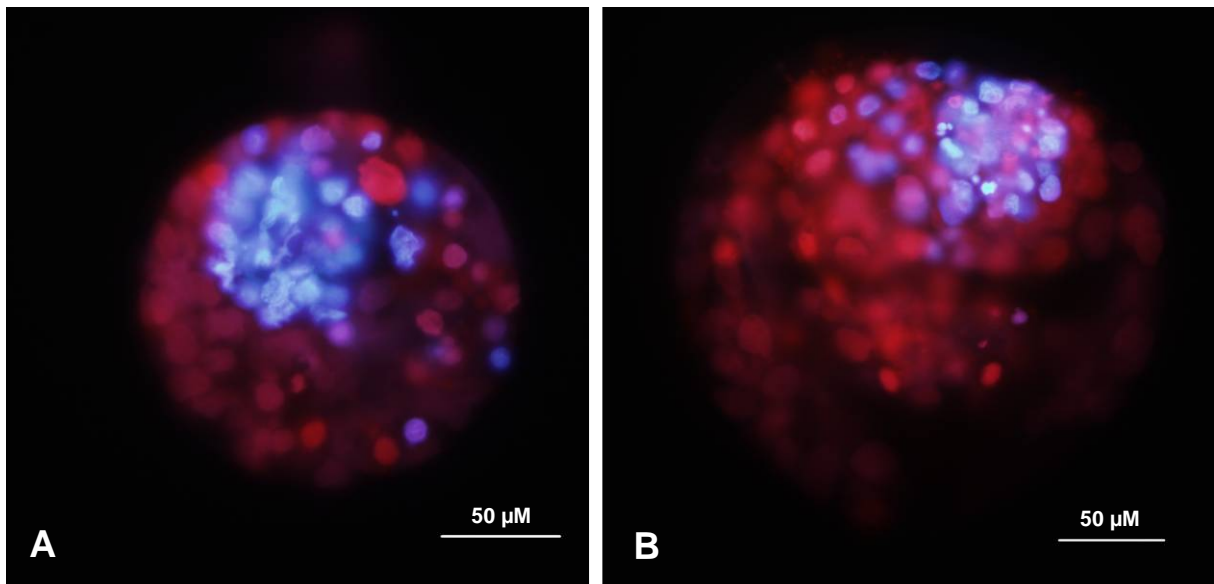


Figure 7.3. Differential staining of day-8 bovine NT (A) and parthenogenetically activated (B) blastocysts with Hoechst 33342 (ICM, blue) and PI (trophectoderm, red) under fluorescence microscopy.

percentage of cells in the ICM from total cell number per blastocyst were different (30% vs. 18%;  $P < 0.05$ ). The highest total number of cells obtained in eland igNT embryos was 23 cells.

#### ▪ DNA Synthesis by BrdU Detection

In this experiment, DNA synthesis during the first cell cycle was evaluated as indicator of early nuclear remodeling after NT. BrdU incorporation was not observed in bovine ( $n=27$ ) and eland ( $n=28$ ) NT cybrids that were not subjected to activation after NT, indicating that these cells did not pass through the S-phase (Figure 7.4). However, when activated cybrids were exposed to BrdU at 4 hpa and cultured for 12 hours, 70% (21/30) and 75% (12/16) of eland and bovine NT cybrids incorporated BrdU into their nuclei, respectively. Similarly, 93% (14/15) of PAOs incorporated BrdU (Table 7.2).

The percentage of nuclei that incorporated BrdU in newly synthesized DNA did not differ ( $P > 0.05$ ) among treatments. The morphological stages of the nuclei of bovine and eland NT cybrids and of PAOs that were fixed 16 hpa were similar. Premature chromosome condensation (PCC), swollen nuclei and cybrids containing two nuclei were observed. No significant differences were found between types of cybrids (Table 7.2 and Figure 7.5). Although 28% eland igNT cybrids and 64% of PAOs were still at the PCC stage at 16 hpa, all of the bovine cybrids have progressed to swollen nuclei or the 2-nuclei stage at 16 hpa.

Of NT embryos that cleaved by 72 hours ( $n=31$ ), 17 of the bovine embryos had  $<8$  cells (55%) and 14 had  $\geq 8$  cells (45%), whereas most eland NT embryos ( $n=20$ ; 87%) had  $<8$  cells and few had  $\geq 8$  cells ( $n=3$ ; 13%; Table 7.2). Nonetheless, the mean number of nuclei between bovine and eland NT embryos were similar. Bovine and eland embryos with  $<8$  cells had an average of  $2.9 \pm 0.3$  and  $3.6 \pm 0.3$  cells, respectively, and those with  $\geq 8$  cells had  $13.0 \pm 1.1$  and  $13.0 \pm 1.3$  cells, respectively. The percentage of cells incorporating BrdU was lower in bovine (3/17; 18%) and eland (6/20; 30%) NT embryos that had  $<8$  cells, than in bovine (14/14; 100%) and eland embryos (2/3; 66%) (Figure 7.6) that had  $\geq 8$  cells ( $P < 0.01$  when species data were pooled).

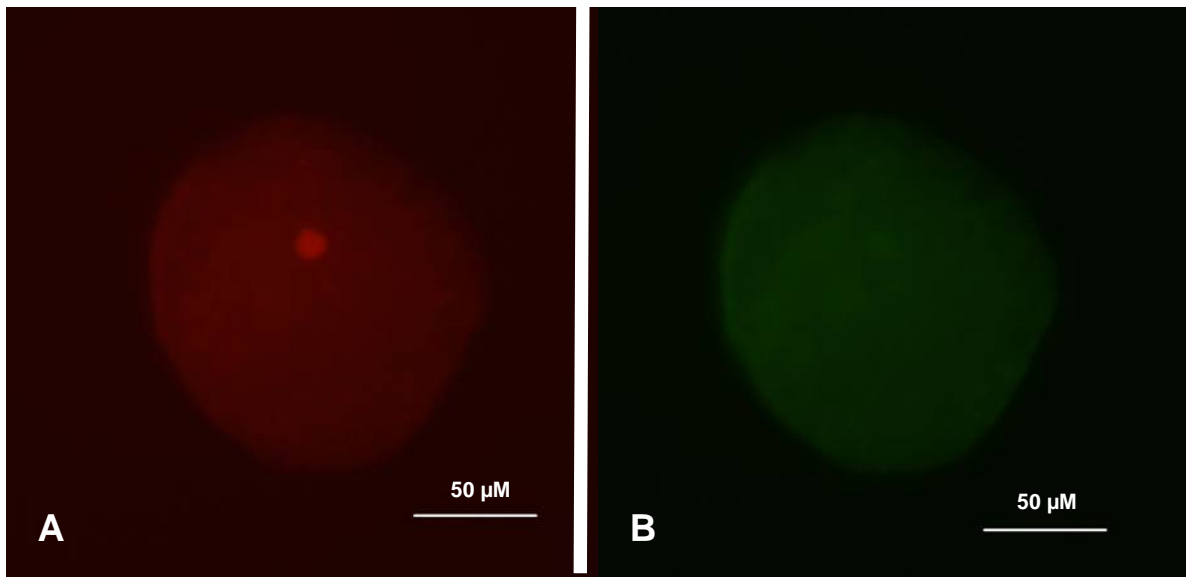


Figure 7.4. Bovine NT cybrid incubated in BrdU prior to activation stained with PI (A) and labeled with FITC-conjugated anti-BrdU antibody (B).

Table 7.2. I: BrdU incorporation of nonactivated bovine and eland NT cybrids (2 hours in BrdU starting at 2 to 3 hours post-fusion).  
 II: BrdU incorporation and nuclei stage of activated bovine and eland NT cybrids and PAOs (12 hours in BrdU starting at 4 hpa).  
 III: BrdU incorporation and cell numbers of cleaved bovine and eland NT embryos and PAOs (12 hours in BrdU starting at 72 hpa).

I. Nonactivated NT cybrids									
	Bovine	Eland							
NT cybrids, n	27	28							
BrdU incorporation	0	0							
II. Activated NT cybrids (4 hpa)									
	Bovine	Eland	Parthenogenetic						
NT cybrids, n	16	30	15						
BrdU incorporation	12/16 (75%)	21/30 (70%)	14/15 (93%)						
Swollen nucleus	0	6/21 (28%)	9/14 (64%)						
PCC	8/12 (66%)	7/21 (33%)	2/14 (14%)						
2-Cell	4/12 (33%)	8/21 (38%)	3/14 (21%)						
III. Cleaved NT embryos (72 hpa)									
Animal type	Total embryos <sup>a</sup> N	2 to 7 cells				≥8 cells			
		PI		BrdU incorporation		PI		BrdU incorporation	
		Embryos n (%)	Mean no. nuclei±SE	Embryos n (%)	Mean no. nuclei±SE	Embryos n (%)	Mean no. nuclei±SE	Embryos n (%)	Mean no. nuclei±SE
Cow	31	17/31 (55)	2.9±0.3	3/30 (10)	3±0.6	14 (45)	13±1.1	14 (100)	12±1.0
Eland	23	20/23 (87)	3.5±0.3	4/20 (20)	3±0.8	3 (13)	14±1.3	2 (66)	14±2.0

<sup>a</sup> Only embryos with at least 2 nuclei shown by PI staining.

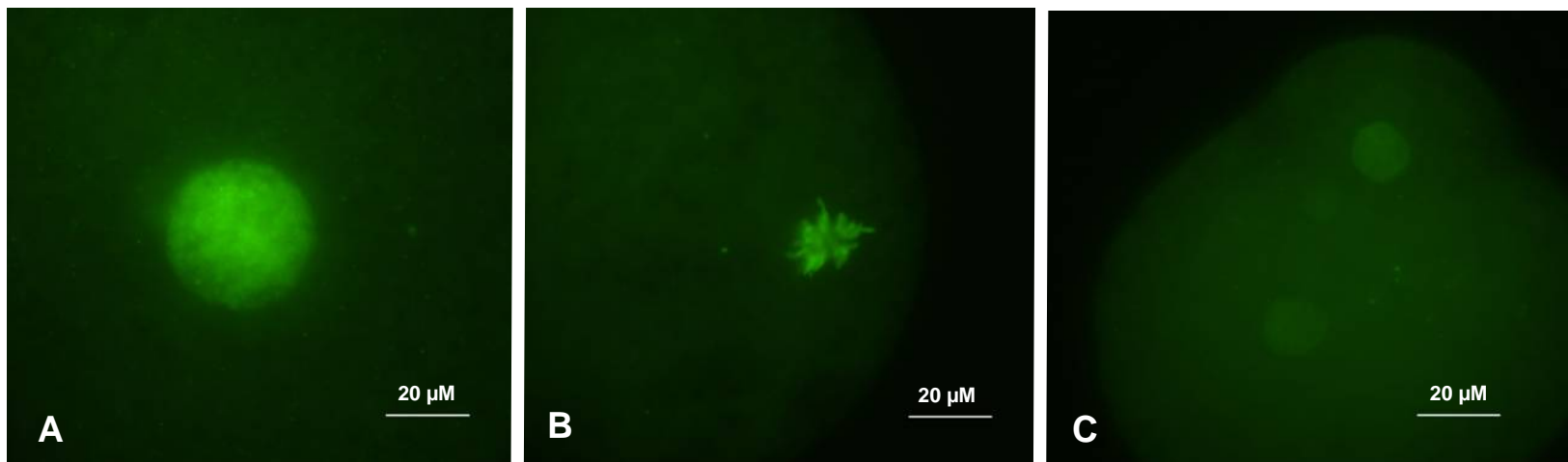


Figure 7.5. Eland embryos (A + B) and bovine embryos (C) labeled with FITC-conjugated anti-BrdU antibody showing a swollen nucleus (A), PCC (B) and two nuclei (C) after culture in BrdU for 12 hours at 16 hours after activation.

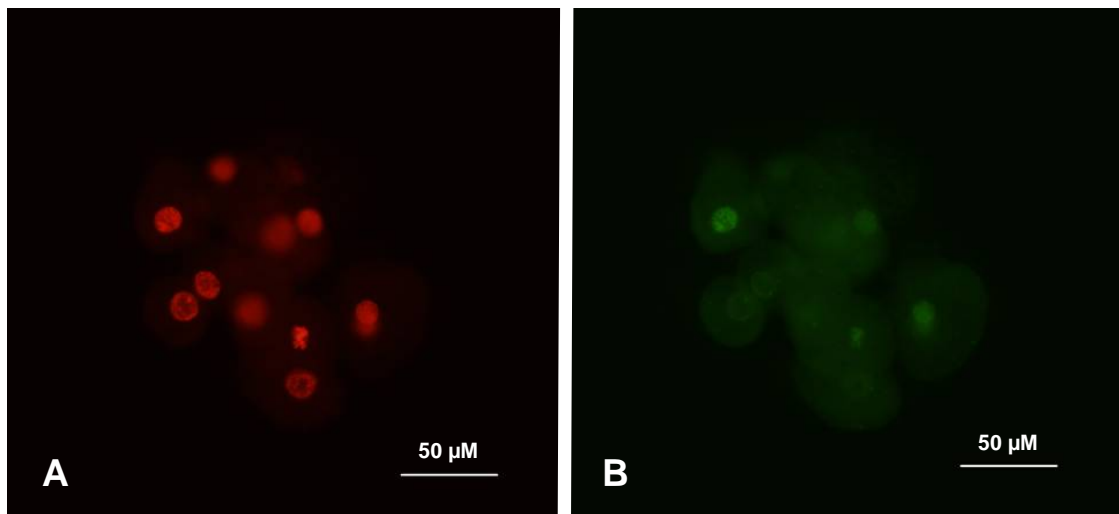


Figure 7.6. PI (A) and BrdU (B) labeling of a 16-cell eland igNT embryo 84 hours after activation showing DNA synthesis in eland igNT nuclei under fluorescence microscopy.



## 7.4 Discussion

In the present study, we demonstrated that nuclei of eland epithelial cells derived from semen can replicate and divide following igNT into enucleated bovine oocytes, synthesize DNA at 16 hpa and cleave at frequencies similar to that of bovine NT embryos. However, igNT eland embryos did not develop to the blastocyst stage and a high proportion of the embryos arrested by the 8-cell stage, which coincides with genomic activation in bovine oocytes (Camous et al., 1984; Kopečný et al., 1989b, 1989a). Although limited data is available on the development of *in vitro* produced eland embryos, intraspecies eland NT embryos also arrested at this stage of development (Matshikiza et al., 2004; Wirtu, 2004).

Cleavage rates of intergeneric cloned embryos created by using bovine oocytes as recipient cytoplasts was similar to the cleavage rates observed by intraspecies bovine NT (Dominko et al., 1999; Lu et al., 2005; Murakami et al., 2005). Also, higher cleavage rates have been reported after the transfer of sheep (66%), monkey (85.7%) and rat (90.2%) somatic cells into cow cytoplasts when compared with the transfer of bovine cells (55.8%) (Dominko et al., 1999). Likewise, in our study, eland igNT and intraspecies bovine NT embryos cleaved at similar frequencies (83% and 80%, respectively), further confirmation that bovine oocytes can induce nuclear reorganization of somatic cells from a different genus. However, even though bovine oocytes can induce early nuclear remodeling, blastocyst development of igNT embryos is usually reduced compared with that seen after intraspecies NT (Matshikiza et al., 2004; Lu et al., 2005; Murakami et al., 2005; Sansinena et al., 2005).

Nevertheless, blastocyst development has been observed when somatic cells of other antelope, such as the bongo (21%; *Tragelaphus euryceros*) and the Giant eland (27%; *Taurotragus derbianus*), were transferred into bovine cytoplasts (Damiani et al., 2003; Lee et al., 2003). In addition, the frequency of blastocyst development after saola (*Pseudoryx nghetinhensis*) (28%) igNT was similar to that of bovine NT (23%) (Bui et al., 2002). Although

saola is classified under the subfamily Bovinae, little is known about the phylogeny of this new species and their relationship to domestic cattle is unclear. The bongo and Giant eland are also members of the Bovinae family and are relatively closely related to Common eland antelope (*Taurotragus oryx*). Interestingly, only one (2%) Common eland igNT embryo reached the blastocyst stage following transfer of Common eland fibroblasts into bovine cytoplasts (Matshikiza et al., 2004) and none developed in the current study. Although comparisons between experiments are confounded by variations in the type of cells, embryo culture systems and criteria used to determine development, a possible explanation for the contrasting results in blastocyst development of antelope NT embryos may be associated with species-specific differences.

Cloned calves can readily be produced after transfer of embryos derived from epithelial cells (Zakhartchenko et al., 1999; Kishi et al., 2000; Miyashita et al., 2002; Gong et al., 2004). However, there are differences between cell lines and cell types in developmental potential of the resulting cloned embryos (Kato et al., 2000; Servely et al., 2003; Batchelder et al., 2005). For example, live offspring have been produced after the transfer of cloned NT embryos derived from mammary epithelial cells, but embryos derived from actively proliferating immortalized mammary epithelial cells failed to develop to the blastocyst stage (Zakhartchenko et al., 1999). The authors suggested that chromatin modifications in some cell types may prevent nuclear reprogramming and disruption of gene regulation caused by *in vitro* culture may also be involved in failure of embryo development. Expression patterns of cells change over time during *in vitro* culture, as exemplified by the report that cytokeratin and vimentin expression in epithelial and fibroblast cells were different in early vs. later passages (Zakhartchenko et al., 1999).

In the present study, we used epithelial cells that were isolated from eland semen and cultured *in vitro* for a prolonged period. While we do not know how *in vitro* culture conditions affected the donor nucleus, some epigenetic modifications possibly influenced nuclear remodeling and reprogramming of derived cloned embryos.

Notably, blastocysts (8.3%) have been obtained after transfer of the same line of eland epithelial cells into *in vivo* matured eland oocytes (Wirtu, 2004). For these reasons, we can surmise that both epigenetic modifications on the somatic cell and species incompatibilities between the eland donor nucleus and bovine cytoplasm probably contributed to the development failure of the igNT eland embryos. The diploid chromosome number (2n) of the Common eland is 31 for males (fused Y-chromosome) and 32 for females (Pappas, 2002), almost half of that of cattle (2n=60). Although the maternal DNA is removed from the oocyte during NT, this chromosomal difference may have implications for cytoplasmic transcription factors that direct early cleavage and activation of the embryonic genome.

It is known that coordination of the cell-cycle stage of the donor nucleus and the recipient oocyte is essential for successful development of reconstructed embryos (Campbell et al., 1993). Our analysis of the cell-cycle distribution demonstrated that the majority of eland epithelial cells (>82%) were in G<sub>0</sub>/G<sub>1</sub> stages and thus, should be compatible with nuclear transfer.

Another factor to consider is the medium used for culturing igNT embryos, which possibly affected embryo development. *In vitro* derived (IVF, ICSI) eland embryos arrested at the 8-cell stage regardless of the culture medium used. Only one and three cloned blastocysts were obtained after culturing embryos in CR1aa medium supplemented with 5% eland serum or alpha-MEM supplemented with 5% bovine calf serum, respectively (Wirtu, 2004). Culture medium is known to affect *in vitro* development of cloned embryos (Heindryckx et al., 2001; Choi et al., 2002). In fact, bongo igNT embryos developed to the blastocyst stage at a higher rate when they were cultured for the first 3 days in hamster embryo culture medium (HECM-6) and the last 5 days in TCM-199 + 10% fetal bovine serum, than when cultured in modified synthetic oviduct medium (m-SOF) (Lee et al., 2003).

During the present experiment, igNT eland embryos were cultured in CR1aa medium that was developed for bovine embryo culture (Rosenkrans and First, 1994), but in our study it

did not support growth of eland embryos beyond the stage of developmental arrest. It is, therefore, likely that improvements in the culture medium would affect development of igNT eland embryos. Further studies are required to understand disparities among cell lines and to comprehend the mechanisms involved in the genetic incompatibility between the donor nucleus and the cytoplasm of reconstructed cloned embryos. Such information should provide significant insight into the mechanisms involved in nuclear remodeling and developmental arrest in embryos.

Mature oocytes contain high levels of maturation promoting factor (MPF), which is responsible for maintaining arrest of oocytes at metaphase-II. Proper activation of fused couplets is required to induce the events that will result in initiation of the first cell-cycle. Results from the BrdU labeling of cybrids demonstrated that DNA synthesis did not take place before activation. Therefore, because most of the transferred nuclei were in  $G_0/G_1$  stages of the cycle, we can assume that the cell cycle of the transferred bovine or eland nucleus does not resume without oocyte activation. Accordingly, when igNT eland and bovine NT cybrids were exposed to BrdU for 4 hours at 16 hpa, 70% and 75%, respectively, of the cybrids synthesized DNA, indicating that MPF activity in the oocyte cytoplasm declined and that DNA synthesis during the first cell-cycle was similar in both types of embryos.

Alberio et al. (2001) reported that 80% of cloned bovine embryos synthesized DNA at 2 hours after removal (5 to 7 hpa) of bohemine. Similarly, in sheep NT embryos, DNA synthesis was initiated ~5 hpa, peaked at ~10 hpa and was completed at 18 hpa (Liu et al., 1997). Although we did not evaluate the exact time at which cloned embryos started DNA synthesis, our results indicated that most bovine NT and eland igNT embryos synthesized DNA by 16 hpa.

Evaluation of nuclear status (swollen nucleus, condensed chromatin or 2 nuclei) in cloned embryos that stained positive for BrdU revealed that 28% of the eland igNT embryos had a swollen nucleus, whereas, all of the bovine NT embryos had progressed to the condensed chromatin stage (66%). In contrast, the number of embryos with 2 nuclei was similar for eland

(38%) and bovine (33%) NT embryos, suggesting that some of the eland igNT embryos were delayed in development. However, these “delayed” embryos did synthesize DNA and the developmental discrepancy may have been a function of the difference in cell types between the two species. It is unclear why the bovine PAOs, despite progressing through the S-phase within 16 hours, progressed more slowly through the different nuclear stages than did the nuclear transfer groups.

Most eland igNT embryos arrested at the 2 to 7-cell stage (87%) and only 20% of them went through DNA synthesis during the 12-hour BrdU incubation. Only 66% (2/3) of the eland igNT embryos that progressed to the 8-cell stage by 84 hpa synthesized DNA in all blastomeres. Likewise, 100% of the cells from  $\geq 8$ -cell embryos in the bovine NT group stained positive for BrdU. Thus, although most igNT embryos arrested earlier than bovine oocytes, a few continued dividing up to 84 hpa, indicating that some igNT embryos were able to progress through the time of the onset of genomic activation. Improvements of the *in vitro* culture system may improve the potential for blastocyst development of these embryos.

Potentially, nuclear transfer technology can have a positive impact on endangered species conservation and the production of elite animals. Semen derived somatic cells could be particularly valuable when semen from deceased animals is available but of limited supply. By producing cloned offspring, the genome of the animal of interest could be introduced into the gene pool by natural breeding and, therefore, aid in reducing the negative consequences of severe inbreeding, which is a concern in zoo populations and heavily managed livestock breeds.

Cloning efforts in endangered species often relies on interspecies nuclear transfer since oocytes from the endangered animal are seldom available. Thus, it is important to further examine the mechanisms involved during nuclear remodeling and reprogramming after igNT to aid in understanding factors that affect these processes. Although the early development of igNT embryos appears to be initially similar to that of NT embryos, subsequent embryonic

development is severely impaired, especially after genomic activation. Nonetheless, with further research, the use of semen-derived somatic cells for NT holds promise as a method for aiding in the conservation of endangered species by incorporating otherwise inaccessible genetic material from previous generations.

## CHAPTER 8

### SUMMARY AND CONCLUSIONS

Advances in ART over the past few decades have stimulated the imagination of scientists world-wide to come up with various uses for their new found skills. Human infertility clinics and livestock production has certainly greatly benefited from these technologies. For example, with the development of AI and semen cryopreservation in cattle, the potential number of progeny from a single sire has been reported to increased from 50 in 1939 to 50,000 in 1979 (Foote, 1981) and continued to increase thereafter. The potential of the biotechnologies as a tool in the battle against biodiversity loss has also motivated reproductive biologists to apply them to endangered species.

Few of the newer assisted reproductive technologies are as successful and efficient as AI and ET. For example, the typical success rate, defined as live births per 100 embryos transferred, for intraspecies NT remains a mere 3 to 4% for domestic species. However, success rates usually increase as our knowledge expands and techniques improve. In nondomestic species, these procedures are much more challenging, mainly because less is known about their reproductive biology and there are limited numbers of animals available. While offspring have been produced following IVF, ICSI and NT in most domestic species, live births have been reported in only 19, two and six nondomestic species for each of the three techniques, respectively. Nonetheless, reproductive biologists studying endangered species continue to work toward making these technologies a realistic option for animal conservation programs.

Biological resource banking plays an important role as a repository of biomaterials for ongoing research and as a backup source of genetic diversity, to be used when new technology is developed and current technology becomes more efficient. Therefore, it is important that we continue to develop techniques specifically for use with endangered species. Accordingly, this

study included a series of experiments that focused on improving particular aspects of the cryopreservation process and utilization of biomaterials. Specifically, we evaluated methods for improving the practicality of established procedures that would enable them to be used for conservation of endangered species populations, both *ex situ* and *in situ*.

The first experiment was aimed at increasing the semen output of rams collected by electroejaculation, while reducing stress by minimizing handling time. Two ejaculates collected consecutively were compared by evaluating quality differences to determine if one ejaculate was more appropriate for certain procedures than the other. To summarize briefly, two ejaculates were collected successfully 10 minutes apart, 3 days a week for 3 weeks, resulting in a total of 74 ejaculates from five rams without a significant loss in semen output or quality. The first ejaculate was higher in volume, concentration and total sperm/ejaculate, while motility before and after cooling and cryopreservation was higher for the second ejaculate. These findings were similar to previous studies in rams (Chang, 1945; Salamon, 1962, 1964; Amir et al., 1968; Jennings et al., 1976; Ollero et al., 1996) and bulls (Pickett et al., 1967; Seidel et al., 1969). Sperm 'survival' rate after cryopreservation was also significantly higher in the second ejaculate, which is considered to be a result of the shorter period of time that spermatozoa spent in the cauda epididymides and/or ampulla portion of the vas deferens.

These results could be extremely useful in cases where only a short period of time is available to collect and preserve the maximal volume of semen from genetically valuable animals. The superior motility and freezability of the second ejaculate may be useful for those *in vitro* procedures requiring fewer, but higher quality spermatozoa (e.g., IVF), whereas, the larger volume and higher concentration of the first ejaculate could be more effective for use in AI. Future experiments may determine the optimal rest period between successive ejaculates for maintaining the superior quality of the second ejaculate, while increasing its volume and concentration.



Conservation efforts often involve field expeditions where researchers may go for extended periods without access to a laboratory, which makes the collection of viable bio-materials rather problematic. Therefore, the second experiment was designed to develop a biopsy freezing method that would be effective, yet practical for use in the field. Although tissues have been successfully cultured *in vitro* after cryopreservation (Taylor et al., 1978; Gray et al., 1995; Silvestre et al., 2002) the methods were designed for laboratory use and required electrically powered equipment and media with biodegradable components. It was demonstrated that tissue biopsies can be effectively cryopreserved in liquid nitrogen after equilibration with DMSO. Furthermore, three different freezing media were effective in preserving viability with different equilibration times and tissue types.

In the next experiment the potential of the body fluids, milk and semen, as noninvasive sources of cells for cryobanking was evaluated. Somatic cells are present in milk (Paape et al., 1966) and semen (Evenson et al., 1983) and cells from both sources have been cultured (Gaffney et al., 1976; Collier et al., 1977; Taylor-Papadimitriou et al., 1977; Phillips et al., 1978). Furthermore, both types of cells can be collected noninvasively. In the current study, cells both from milk (sheep) and semen (sheep and eland) were successfully cultured following a simple washing procedure. However, the technique resulted in loss of potentially valuable spermatozoa during culture. Milk cells grown in culture were either fibroblast, epithelial or a mixture of cell types and nearly 40% of the culture attempts produced proliferating cell lines. The ability of fresh, cooled and cryopreserved (-1°C/minute or -10°C/minute cooling rate) ram semen samples to produce proliferating cell lines in culture was evaluated. Initial cell proliferation occurred after fresh and cooled semen samples were placed in culture, but proliferation did not continue after passage. The cryopreservation protocol may possibly be severely damaging to somatic cells and since semen-derived cells were of epithelial origin, the culture system did not support cell proliferation. Eland semen, however, resulted in two proliferating cell lines, which were

subsequently used as karyoplast donors for NT into eland oocytes. Eland NT resulted in blastocysts (12%), but no pregnancies resulted after they were transferred into eland recipients.

The purposes of the next experiment was (1) to develop a method of separating somatic cells from spermatozoa in ram and eland semen samples before *in vitro* culture and (2) to improve the *in vitro* growth and proliferation of the semen-derived somatic cells. In preliminary trials, several separation techniques were evaluated, including the use of filters with pore sizes larger than spermatozoa but smaller than somatic cells. After these efforts were largely unsuccessful at separating the desired cells, it was decided to examine the effectiveness of separating sperm cells from somatic cells by gradient centrifugation.

Methods for separating cell types in mixed suspensions of various origins using Percoll gradient centrifugation have been described (Salisbury et al., 1979; Giddings et al., 1980; Olofsson et al., 1980; Segal et al., 1980; Goust et al., 1981; Riding et al., 1981; Smedsrod et al., 1985). Since the extent of separation is determined by several interactive factors including duration and speed of centrifugation and number and density of each layer of the gradient, it was necessary to determine the specific factors for obtaining optimal separation of the somatic cells from the sperm cells. We found that with a four-layer Percoll gradient, ram and eland somatic cells, as well as motile and nonmotile spermatozoa, could be successfully separated, with epithelial cells settling in the 20% Percoll fraction.

Subsequently, a co-culture system was developed using 3T3 mouse fetal fibroblasts that significantly improved *in vitro* proliferation of semen-derived epithelial cells from both species. Our results were in agreement with previous experiments showing that co-culture with 3T3 fibroblasts improved proliferation of several types of epithelial cells (Rheinwald et al., 1975; Taylor-Papadimitriou et al., 1977; Gray et al., 1983; Lee et al., 1984; Sun et al., 1984; van Blitterswijk et al., 1986; Kabalin et al., 1989). The results of the present experiment also indicated that contact between the feeder cells and epithelial cells was not essential for the improvement in proliferation. It is therefore likely that preconditioned medium may have the

same proliferative effect on the epithelial cells without the risk of fibroblast contamination. Therefore, future studies with conditioned medium would be beneficial. These results also confirmed the results of our earlier experiments in which the semen-derived cells were found to be of epithelial type, although different morphological types of epithelia were observed.

Finally, we examined the ability of eland semen-derived epithelial cells to direct early embryonic development after fusion with bovine cytoplasts. Epithelial cells have been used to produce NT blastocysts and offspring (Zakhartchenko et al., 1999; Kishi et al., 2000; Miyashita et al., 2002; Gong et al., 2004). Furthermore, Lee et al. (2003) reported that blastocysts were produced by igNT of fibroblasts from bongo antelope, a close relative of eland antelope, into enucleated bovine oocytes.

In Chapter 5, blastocysts were derived by intrageneric NT of eland semen epithelial cells into eland oocytes. In the present experiment, however, no igNT blastocysts were produced and it is thought that the combination of cell type, intergeneric effects and possibly a suboptimum culture system for eland embryos may have contributed to the failure. We also determined by BrdU-incorporation that, although DNA synthesis appeared to be similar in igNT and bovine NT embryos at 16 hpa, DNA synthesis in igNT embryos ceased at the ~8-cell stage so that positive labeling for BrdU was minimal at 84 hpa. The authors attribute this to a developmental block at approximately the 8-cell stage, which is concurrent with the block in bovine cytoplasts (Camous et al., 1984; Kopecny et al., 1989a, 1989b). Additional research of igNT, including the role of cell type during NT and modification of the culture system for eland embryos, may increase the possibility of producing developmentally competent igNT embryos.

In conclusion, a series of experiments were conducted to examine methods for improving the practicality of or increasing the efficiency of obtaining, culturing and preserving somatic cells that would be applicable to aiding in the conservation of endangered species. Clearly, the techniques require much improvement. However, based on progress made to date, it is expected that over time, the scope and efficacy of reproductive techniques will continue to

expand. Then, with proper use, these ART, in conjunction with other associated techniques for enhancing reproduction, could play an important role in global efforts to prevent the extinction of species.

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## APPENDIX A

### MEDIUM COMPOSITIONS

#### A1: Dulbecco's Modified Eagle's Medium (Gibco Cat. no. 10313-021)

Components	Molecular weight	Concentration (mg/L)	Molarity (mM)
<u>Amino Acids</u>			
Glycine	75	30	0.400
L-Arginine hydrochloride	211	84	0.398
L-Cystine 2HCl	313	63	0.201
L-Histidine hydrochloride-H <sub>2</sub> O	210	42	0.200
L-Isoleucine	131	105	0.802
L-Leucine	131	105	0.802
L-Lysine hydrochloride	183	146	0.798
L-Methionine	149	30	0.201
L-Phenylalanine	165	66	0.400
L-Serine	105	42	0.400
L-Threonine	119	95	0.798
L-Tryptophan	204	16	0.0784
L-Tyrosine disodium salt dihydrate	261	104	0.398
L-Valine	117	94	0.803
<u>Vitamins</u>			
Choline chloride	140	4	0.0286
D-Calcium pantothenate	477	4	0.00839
Folic acid	441	4	0.00907
i-Inositol	180	7.2	0.0400
Niacinamide	122	4	0.0328
Pyridoxine hydrochloride	206	4	0.0194
Riboflavin	376	0.4	0.00106

(table continued)

Thiamine hydrochloride	337	4	0.0119
<u>Inorganic Salts</u>			
Calcium chloride (CaCl <sub>2</sub> ) (anhydrous)	111	200	1.80
Ferric nitrate (Fe(NO <sub>3</sub> ) <sub>3</sub> •9H <sub>2</sub> O)	404	0.1	0.000248
Magnesium sulfate (MgSO <sub>4</sub> ) (anhydrous)	120	97.67	0.814
Potassium chloride (KCl)	75	400	5.33
Sodium bicarbonate (NaHCO <sub>3</sub> )	84	3700	44.05
Sodium chloride (NaCl)	58	6400	110.34
Sodium phosphate monobasic (NaH <sub>2</sub> PO <sub>4</sub> •H <sub>2</sub> O)	138	125	0.906
<u>Other Components</u>			
D-Glucose (dextrose)	180	4500	25.00
Phenol red	376.4	15	0.0399
Sodium pyruvate	110	110	1.000

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**A2: Minimum Essential Medium Alpha Medium (Gibco Cat. no. 12561-056)**

Components	Molecular weight	Concentration (mg/L)	Molarity (mM)
<u>Amino Acids</u>			
Glycine	75	50	0.667
L-Alanine	89	25	0.281
L-Arginine	211	105	0.498
L-Asparagine•H <sub>2</sub> O	150	50	0.333
L-Aspartic acid	133	30	0.226
L-Cysteine hydrochloride•H <sub>2</sub> O	176	100	0.568
L-Cystine 2HCl	313	31	0.0990
L-Glutamic Acid	147	75	0.510
L-Glutamine	146	292	2.00
L-Histidine	155	31	0.200
L-Isoleucine	131	52.4	0.400
L-Leucine	131	52	0.397
L-Lysine	183	73	0.399
L-Methionine	149	15	0.101
L-Phenylalanine	165	32	0.194
L-Proline	115	40	0.348
L-Serine	105	25	0.238
L-Threonine	119	48	0.403
L-Tryptophan	204	10	0.0490
L-Tyrosine disodium salt	225	52	0.231
L-Valine	117	46	0.393
<u>Vitamins</u>			
Ascorbic acid	176	50	0.284
Biotin	244	0.1	0.000410
Choline chloride	140	1	0.00714

(table continued)

D-Calcium pantothenate	477	1	0.00210
Folic acid	441	1	0.00227
i-Inositol	180	2	0.0111
Niacinamide	122	1	0.00820
Pyridoxal hydrochloride	204	1	0.00490
Riboflavin	376	0.1	0.000266
Thiamine hydrochloride	337	1	0.00297
Vitamin B12	1355	1.36	0.00100
<u>Inorganic Salts</u>			
Calcium chloride (CaCl <sub>2</sub> ) (anhydrous)	111	200	1.80
Magnesium sulfate (MgSO <sub>4</sub> ) (anhydrous)	120	97.67	0.814
Potassium chloride (KCl)	75	400	5.33
Sodium bicarbonate (NaHCO <sub>3</sub> )	84	2200	26.19
Sodium chloride (NaCl)	58	6800	117.24
Sodium phosphate monobasic (NaH <sub>2</sub> PO <sub>4</sub> •H <sub>2</sub> O)	138	140	1.01
<u>Other Components</u>			
D-Glucose (dextrose)	180	1000	5.56
Lipoic acid	206	0.2	0.000971
Phenol red	376.4	10	0.0266
Sodium pyruvate	110	110	1.000

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(www.invitrogen.com)

# APPENDIX B

## LETTER OF PERMISSION



March 29, 2006

Our ref: Nel-ThemaatThesisML4-06

Liesl Nel-Themaat  
Louisiana State University  
616 Memorial Heights Drive, Apt. 9214,  
Houston, TX 77007  
lnell@lsu.edu

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## **VITA**

Liesl Nel, daughter of Etienne and Carin Nel, was born in South Africa on October 26<sup>th</sup> 1977. She attended Sentrale Volksskool and Kroonstad High School in the Free State, South Africa and received her Bachelor of Science degree from Stellenbosch University in 2000. Following completion of the degree, she moved to London, United Kingdom, where she worked as a DNA analyst at the London General Chemistry Laboratories for 7 months, before entering the graduate school at Louisiana State University as a doctoral student under the direction of Dr. Robert Godke.

Liesl married Johan ver Loren van Themaat and made Nel-Themaat her professional last name. She has a very strong interest in the conservation of endangered species through assisted reproductive technologies and therefore, made that the focus of her research. She is scheduled to graduate with the degree of Doctor of Philosophy in May of 2006.